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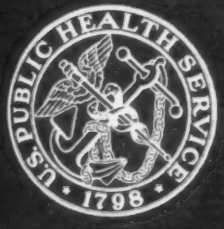

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Histochemical Demonstration of Sulfhydryl and Disulfide Groups of Protein[1,2]

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A new specific method for the histochemical detection of protein-bound sulfhydryl groups was described by us previously (*1*, *2*) and a survey was made of the distribution of these thiols in the tissues of albino rats (*3*). The method was dependent upon the specific reactivity of the reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD),[4] with thiols. In order to modify the sulfhydryl method for the demonstration of disulfide groups as well, a means of reducing disulfide to sulfhydryl was needed. To demonstrate disulfide groups alone, a means of irreversibly blocking the sulfhydryl groups before reducing the disulfide groups was required. The present communication deals with procedures for accomplishing both types of demonstrations in tissue sections as already reported (*1*, *4*, *5*). In addition, an independent, although less specific, method for demonstrating both sulfhydryl and disulfide groups is given. This method is based on the oxidizing properties of ditetrazolium chloride (BT)[5] at elevated *p*H (*1*, *6*, *7*). Water-insoluble blue diformazan is formed at the sites of strong reducing groups (thiols).

Experiments are also included which demonstrate the specificity of the methods developed. In addition, a study was made of various means of fixing tissues so as to retain proteins that are ordinarily soluble in acid alcohol. The significance of the differences obtained by such modifications of the procedure is discussed. A survey of the sites of sulfhydryl

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and disulfide in tissues of the rat and other animals is presented and the significance of some of these results is also discussed.

Materials and Methods

Materials.—Tissues of freshly killed adult albino rats were principally used. In addition, a few tissues were obtained from man, monkey, cat, dog, armadillo, hamster, mouse, deer, ox, chicken, and lamprey eel. The tissues were routinely fixed for 24 hours in a solution of 1 gm. of trichloroacetic acid dissolved in 100 cc. of 80 percent ethyl alcohol (acid alcohol). They were then dehydrated, embedded in paraffin, sectioned at 5 to 10 μ, and deparaffinized. A minimum of albumin was used in mounting the sections on slides. Hard tissues, such as feathers and claws, were embedded in butyl methacrylate after fixation in acid alcohol, and sectioned at 4 μ or less with either a glass or a steel knife. The methacrylate was removed from the sections with either toluene or isoamyl alcohol and the sections were passed through each step in the reactions for sulfhydryl alone or sulfhydryl and disulfide before they were mounted. This procedure proved unnecessary for lamprey teeth, which could be cut after paraffin embedding.

Fixation.—The results with other fixatives were explored also since it was expected that certain proteins soluble in the acid-alcohol fixative would be retained in tissue sections. The fixatives used were Zenker's fluid, Zenker-formol, 10 percent neutral formalin, 10 percent formalin in saturated mercuric chloride, Bouin's fluid, Carnoy's fluid, phosphotungstic acid, phosphomolybdic acid, and sulfosalicylic acid. Although Zenker's fluid which was frequently used in these experiments gave excellent results in preserving both sulfhydryl and disulfide groups, free sulfhydryl groups alone were poorly demonstrated after this fixative, since dichromate or subsequent treatment with Lugol's solution (U.S.P.) oxidized sulfhydryls to disulfides, and mercuric chloride formed mercaptides. Therefore, Zenker's fluid was used as a fixative only for the demonstration of both sulfhydryl and disulfide groups. It was not possible to determine by inspection of the sections alone whether one or both of the groups were responsible for the final staining reaction. Although this imposed a severe limitation on the method, worth-while results were obtained in certain tissues where significantly larger amounts of reactive protein were retained in the tissue after fixation with Zenker's fluid than with acid alcohol. Whenever other fixatives were used, controls were always carried out with tissue fixed in acid alcohol and stained for either sulfhydryl or for both sulfhydryl and disulfide groups.

The use of formalin as a fixative is not without hazard because of its reactivity with sulfhydryl and disulfide groups (*8*). However, when dilute neutral formalin was used, and when the tissues were washed thoroughly with water, the staining obtained was quite satisfactory compared to that with the other fixatives. Reactivity of these groups with formalin at room temperature and neutrality may be less extensive than previously

thought (*8*), or the reaction products may be dissociated by prolonged washing (*9*).

Zenker-formol and Bouin's fluid were not quite as satisfactory as Zenker's fluid or Carnoy's fluid. For instance, the protein picrates formed with Bouin's fluid and some pure proteins *in vitro* were more soluble in 80 to 90 percent alcohol than the protein complex formed with Zenker's fluid (*10*). No complication was introduced by mercaptide formation with mercuric chloride in demonstrating disulfide and sulfhydryl together, because the mercaptides formed were readily dissociated by the reducing agents (thiols) and by the high hydroxyl ion concentration that was necessary for reducing disulfide groups. An important property of mercaptides is their reversibility under these conditions (*11*). Phosphomolybdic, phosphotungstic and sulfosalicylic acids (*12*) were good fixatives for the demonstration of disulfides of protein soluble in other fixatives.

Selection of the reducing agent.—Many reducing agents were tested for their effectiveness in splitting disulfide linkages in sections of a variety of tissues and in pure proteins *in vitro* (*10*). Most of the agents reduced all disulfides if used in sufficient concentration at high *p*H and if the exposure was sufficiently prolonged. However, limitations were imposed by the distortion and destruction of tissue sections which some of these conditions produced, and by the solvent action of some of the reducing agents on proteins. Therefore, in order to preserve good histologic structure it was necessary to accept conditions that varied somewhat for different tissues, depending on their composition and ability to withstand the treatment, and that resulted in incomplete reduction in many cases. Some tissues that contain keratins, such as skin, were able to withstand more drastic treatment than others. Although conditions were found that resulted in a satisfactory histochemical demonstration of these functional groups, it is important to emphasize that quantitation was not considered possible.

The following reducing agents were tested: hydrogen sulfide and its salts, sodium bisulfite, sodium hydrosulfite, sodium thiosulfate, L-ascorbic acid, L-epinephrine, potassium cyanide, sodium thioglycolate, cysteine, glutathione, 2-mercaptoethanol, and 2,3-dimercaptopropanol (BAL). The last two were used in solutions in absolute acetone containing a few drops of pyridine, whereas the others were used in aqueous solutions at *p*H 8 to 11.5. Various concentrations of reducing agents and exposure times were used between 37° and 50° C. up to the tolerance of the tissue. The sections of tissue were covered with a thin, protective layer produced by the application of ½ percent celloidin before exposure to the reducing agents. After reduction, the sections were washed for 10 minutes in running tap water, briefly in water acidified with acetic acid (1 percent), and again in tap water. The acid rinse inactivated the reducing agents, facilitated their complete removal from the tissues, and at the same time prevented air-oxidation of the newly formed sulfhydryl groups. In the case of the organic reductants, 2-mercaptoethanol and BAL, alcohol and acid-alcohol rinses were used for the same purposes.

Of these reducing agents, sodium thioglycolate, ammonium sulfide, potassium cyanide, and sodium hydrosulfite ($Na_2S_2O_4$) were, in decreasing order, most useful. Although BAL was very effective, it was expensive and inconvenient to use. Sodium hydrosulfite was effective only at a concentration (12 M) and *p*H (10.5) that were markedly destructive of tissue, but it was useful in control experiments as a powerful reducing agent for comparison with sulfhydryl-containing reducing agents. Potassium cyanide (10 percent) not only required a high *p*H (11 to 11.5) and a long period of incubation, but this relatively weak agent produced, by addition, only one sulfhydryl group from disulfide together with an inert thiocyanate group (*9*, *13*). These considerations were borne out by the lesser intensity of staining in sections treated in this manner.

Ammonium sulfide (5 percent) was a powerful, inexpensive, convenient reducing agent that produced particularly intense staining reactions due to its reducing power, solvent action, and penetrating ability. However, when an occasional structure such as elastic tissue, which was stained only slightly or not at all when other reducing agents were used, was intensely stained after ammonium sulfide treatment, we became suspicious that artefacts were being produced by the addition of the ammonium sulfide to reactive groups such as carbonyl to form new, fixed sulfhydryl groups. This was established by two types of experiments. In one, carbonyl groups were irreversibly blocked by treatment of the sections with semicarbazide for 1 to 2 hours at 50° C. Subsequent reduction with ammonium sulfide resulted in a significantly weaker stain than when parallel sections were treated only with ammonium sulfide. In other experiments, aldehyde groups were produced from the polysaccharides in sections of liver by oxidation with periodic acid. When these sections were treated with ammonium sulfide, additional sulfhydryl groups were demonstrable, whereas when similar sections were treated with the reducing agent sodium thioglycolate, additional sulfhydryl groups were not demonstrable compared with unoxidized sections. Furthermore, when periodic acid–oxidized sections of liver or of elastic tissue were treated with the irreversible carbonyl-blocking agent potassium cyanide, subsequent treatment with ammonium sulfide did not produce more sulfhydryl groups than were noted after treatment with sodium thioglycolate.

Although these experiments proved that ammonium sulfide can produce demonstrable sulfhydryl groups in the rare sites of active carbonyl groups, since lipid which is ordinarily the commonest source of carbonyl groups is removed in the preparation of the sections, artefacts such as in elastic tissue are rare. Perhaps this most effective reductant should not be discarded, since it works rapidly at a relatively low alkaline *p*H (9.5) and therefore causes less tissue distortion than the others. However, it must be used with suitable controls when exploring new tissues.

Although sodium thioglycolate was not the strongest reducing agent, it was the best reducing agent that was also specific for conversion of disulfide to sulfhydryl (*9*). It is, however, 50 times more effective than

cysteine (*14*) or cyanide (*9*) in producing reduced disulfides. The intensity of staining after its use was satisfactory, although frequently not as great as with ammonium sulfide or BAL.

With the exception noted for ammonium sulfide, the distribution of the staining reaction (but not the intensity) was similar if not identical with all the reducing agents used.

Procedure for Demonstrating Both Sulfhydryl and Disulfide Groups

Deparaffinized sections of tissue, covered with a thin layer of ½ percent celloidin that was allowed to evaporate, were incubated for 1 to 2 hours at 50° C. in 0.2 M to 0.5 M fresh thioglycolic acid titrated to *p*H 8 with 0.1 N sodium hydroxide just before use. The sections were washed in running tap water for 10 minutes, rinsed briefly in 1 percent acetic acid, and finally in tap water again. The sections were then immediately treated with the reagents (DDD and tetrazotized diorthoanisidine) according to the procedure for sulfhydryl described in detail earlier (*2*).

Procedure for Demonstrating Disulfide Groups

Deparaffinized sections of tissue were incubated for 20 hours at 37° C. in 0.1 M iodoacetic acid titrated to *p*H 8 with 0.1 N sodium hydroxide, or for 4 hours at 37° C. in 0.1 M N-ethyl maleimide [4] (*15*) in 0.1 M Sorensen's phosphate buffer at *p*H 7.4. After being washed in 1 percent acetic acid and running tap water, representative tissue sections failed to react for sulfhydryl groups. The remaining sections were then incubated in 10 percent potassium cyanide for at least 2 hours at 37° C. The sections were thoroughly washed in running tap water for 10 minutes and stained for sulfhydryls with DDD (*2*). It was necessary to use the weakest disulfide-splitting agent in this procedure because the more powerful reductants removed the blocking groups on sulfhydryls, thereby allowing the demonstration of both sulfhydryl and disulfide rather than disulfide alone.

Ditetrazolium Chloride (BT) Method for Demonstrating Both Sulfhydryl and Disulfide Groups

Deparaffinized sections of tissue were covered with celloidin as before and were incubated for 8 to 12 hours in 48 cc. of 10 percent potassium cyanide to which had been added 2 cc. of 1 N sodium hydroxide and 25 mg. of BT (*6*, *7*). Although alkali or potassium cyanide could be used alone for splitting disulfide (*9*, *12*), the reaction proceeded faster when both were used. The sections developed a blue pigment in the course of the reaction. They were washed in running tap water and a cover glass was mounted with Kaiser's glycerogel. Neotetrazolium chloride (*16*) was found to react even faster than BT so that the incubation time could be reduced to 2 to 3 hours, and it produced a purplish-red pigment instead of the blue pigment obtained from BT.

[4] See footnote 4, page 769.

Specificity of the Histochemical Procedures

Evidence for the specificity of the reaction of DDD (2,2′-dihydroxy-6,6′-dinaphthyl disulfide) with sulfhydryl groups was given in detail elsewhere (*2*, *3*). Some of these experiments were repeated here to show that the modifications introduced into the original method to demonstrate disulfide after splitting to sulfhydryl had not resulted in loss of specificity. Most of these experiments were performed with sections of rat skin because this tissue is rich in both sulfhydryl- and disulfide-containing proteins.

Specificity of the Procedure for Both Free Sulfhydryl and Sulfhydryl Obtained From Disulfide

When tissue sections were oxidized for 4 hours with an aqueous solution of 38 mg. of iodine and 33 mg. of potassium iodide in 100 cc. of water, titrated to *p*H 3.2 with 0.01 N hydrochloric acid, no histochemical reaction for sulfhydryl could be obtained. Under these conditions it is claimed that sulfhydryl groups are specifically oxidized (*17*, *18*). That the oxidation had not proceeded further than to disulfide was shown in experiments in which iodine-treated sections were reduced with sodium thioglycolate, ammonium sulfide, BAL, or potassium cyanide, and subsequently stained for sulfhydryl. These results were similar to those in which sections had not been exposed to iodine, but were reduced and stained as above. However, iodine treatment after reduction of disulfide again blocked the sulfhydryl staining reaction, as did treatment with 0.1 M iodoacetate at *p*H 8 for 20 hours at 37° C. or with 0.1 M N-ethyl maleimide at *p*H 7.4 for 4 hours at 37° C.

In another kind of experiment it was shown that the reaction product formed by DDD and protein-sulfhydryl could be reversed by an excess of a sulfhydryl-containing compound. When sections with sulfhydryl groups (both free and those produced by reduction of disulfides) had reacted with DDD, and before the pigment was produced by coupling with tetrazotized diorthoanisidine, incubation with 0.03 M glutathione at *p*H 8.5 for 3 hours at 50° C. completely blocked coupling to the azo dye by reversal and removal from the sections of the sulfhydryl-bearing naphthol moiety that was demonstrated in parallel sections. This experiment emphasizes the specificity of the oxidation-reduction that exists between sulfhydryl and disulfide groups (*9*, *11*, *19*, *20*). Attempts to remove the final colored product from tissue sections by the combined use of reducing agents and solvents failed because of the insolubility of the final product due to cross-linking by the coupling agent.

Specificity of the Procedure for Sulfhydryl Obtained From Disulfide

The same experiments were performed after blocking sulfhydryl and opening disulfide groups. The histochemical demonstration of these groups was also prevented by treatment with iodine, iodoacetate, or N-ethyl maleimide, and their reaction with DDD was reversed by treatment with glutathione.

Comparisons of sections stained for 1) sulfhydryl, 2) sulfhydryl and disulfide, or 3) disulfide alone revealed that the difference in intensity of stain between 1) and 2) was about equal to that of 3) in tissues where all the staining occurred at the same sites. Differences in intensity and distribution occurred when sulfhydryl and disulfide occurred at different sites (*i.e.*, nerve and hair).

Specificity of the Procedure With Ditetrazolium Chloride

Since the tetrazolium salts are reducible by other functional groups (*7*) as well as sulfhydryl, methods based on their use do not have any specificity. The high alkalinity required for inducing the reaction also limits their use to fairly rugged tissues, such as epidermis and hair. In addition, this tissue provided an adequate base-line for comparison since the localization of sulfhydryl and disulfide have been studied extensively by a large number of methods (*3*, *13*, *21–24*).

Since it was expected that the high *p*H required for the reaction would result in extensive cleavage of disulfide (*13*, *25*), potassium cyanide was added to facilitate the cleavage of disulfide when it was shown that cyanide alone did not reduce BT, and it was accepted that both sulfhydryl and disulfide groups would be demonstrated simultaneously by this method.

Evidence for the specificity of this reaction was based largely on ruling out the presence of other substances that would also reduce tetrazolium salts. Reductants such as glucose, ascorbic acid, glutathione, and epinephrine are water-soluble and would be extracted during fixation in alcohol–trichloroacetic acid. With the exception of glutathione, these compounds reduce tetrazolium salts at a *p*H lower than that required to give a reaction with skin. Lipoidal aldols and ketols would have been eliminated during the process of embedding in paraffin. Sections of skin treated with the sodium hydroxide–potassium cyanide mixture without tetrazolium salts did not stain by the Ashbel-Seligman reagent (*26*) or by the Schiff reagent without previous exposure to periodic acid.

Further evidence was provided in experiments in which sections were oxidized with iodine. These sections stained just like those which had not been treated with iodine. Furthermore, sections treated with sodium hydroxide–potassium cyanide, then oxidized with iodine, and finally resubjected to the sodium hydroxide–potassium cyanide–tetrazolium salt mixture also demonstrated the same localization of the histochemical reaction. We are not aware of substances in tissues, containing groups other than sulfhydryl, that can be reversibly oxidized with iodine and restored by reduction with alkaline cyanide. In skin, the histochemical localization with this agent was the same as with the method for both sulfhydryl and disulfide, which employs the specific reagent DDD. The results with other tissues should be used only after similar control studies.

The reduction of tetrazolium salts appeared to be caused by sulfhydryls and not by hydrogen sulfide, which may be produced by the action of alkali on disulfide (*13*). This was indicated by production of the same

histochemical result after sections were incubated in the alkali-cyanide-tetrazolium mixture as when sections were first incubated in alkali-cyanide, washed, and reincubated in the alkali-cyanide-tetrazolium mixture.

Histochemical Localization of Disulfide Groups

Although disulfide groups of protein were widespread in tissues, the present survey is restricted to selected tissues to indicate the over-all results of a larger sampling. The histochemical localization of certain specific disulfides in tissues will be published elsewhere (*27–29*). With some exceptions, as will be specifically noted below, disulfide groups of proteins were frequently present in cells that contained protein-bound sulfhydryls. In some instances, the intracellular localization of disulfide appeared to be the same as that of sulfhydryl, and these cells merely stained more intensely for sulfhydryl and disulfide groups than for sulfhydryl alone. Occasionally, however, disulfides were localized in distinguishably different intracellular sites, and in some instances cells that were negative for sulfhydryl were positive for disulfide. Some tissues contained no demonstrable disulfide, although they were positive for sulfhydryl. As in the case of sulfhydryl, cell nuclei contained little disulfide in comparison with the cytoplasm. Unless otherwise mentioned, the following description of the sites of disulfide groups applies to sections of rat tissue fixed with alcohol–trichloroacetic acid, stained for both sulfhydryl and disulfide after thioglycolate reduction, and compared with sections stained for sulfhydryl alone. Since the work on the distribution of sulfhydryl groups in rat tissues has been described elsewhere in detail (*3*), such observations will not be repeated except in those instances where the material was not previously covered.

Epidermis and hair.—It was difficult to determine whether disulfide groups were present in the epithelial cells of the stratum Malpighii, observed in sections of skin taken from the dorsum of rats and from other animals (armadillo, deer, hamster, mouse, monkey, and man). With the combined sulfhydryl and disulfide method, there appeared to be a minimal intensification of the reaction over that seen in sections stained for sulfhydryl alone in outer parts of the stratum Malpighii (figs. 11, 13). In the sections stained for disulfide alone, no reaction was observed at these sites (fig. 15). This may have been due to the production of fewer reactive groups with the latter method. The reaction for disulfide in the stratum granulosum was positive but weak, regardless of whether the reaction for sulfhydryl was strong (as in the skin of the rat shown in figure 10 and the epidermis of the armadillo shown in figure 18), or was weak (as in the rat skin shown in figures 11, 13, 15). The keratohyaline granules in the stratum granulosum were unreactive. The strongest reaction for disulfide groups in the epidermis was present in the stratum corneum (figs. 10, 11, 13, 15). However, it should be emphasized that the reaction there was only weak or moderate, even in sections that were subjected to strenuous treatment with reducing agents.

Mature resting hair revealed an intense reactivity for disulfides of the keratinized hair cortex in the outer one-half to two-thirds of the hair shaft, a region which was completely negative for sulfhydryl (figs. 10, 11, 13, 15). This staining was unequaled in intensity by any other rat tissue. The reaction for disulfide was less intense and was scattered in some of the substance of the keratogenous zone and the region just proximal to it (fig. 16), an area that was most intensely reactive for sulfhydryl (fig. 14). The reaction for disulfide appeared to be localized in the fibrillar substance of the hair cortex. This substance appeared to arise from a precursor which was a product of cells distal to the hair bulb and was positive for sulfhydryl (fig. 14).

In the remainder of the structures comprising the mature hair, the reaction was by no means as striking. The medulla throughout its extent appeared free of disulfide (figs. 10, 11). The cuticle was weakly reactive. The entire inner root sheath, including sheath, Huxley's layer with the trichohyalin granules, and Henle's layer, was for the most part unreactive (figs. 14, 16). The outer root sheath reacted weakly at best (fig. 13). The papillae and the cells forming the matrix in the bulb were completely unreactive (fig. 16). Both cells and fibers of the connective tissue of the dermis were negligibly reactive under ordinary conditions of staining (figs. 10, 11, 13, 15). After excessive reduction, a diffuse but weakly positive reaction occurred in the connective tissue, but it was impossible to localize the staining clearly in either collagen fibers or ground substance. However, under these conditions, the connective-tissue cells were positive. In the epithelial cells of the sebaceous glands and in the arrectores pilorum muscles (fig. 13) little disulfide was noted.

In sections fixed in Zenker's fluid and subjected to the method for both sulfhydryl and disulfide, there was an over-all increase in the intensity of staining of most of the elements of skin except the cortex and medulla of the hair. The hair papilla, matrix, root sheaths, epidermis, and connective-tissue fibers and cells of the dermis were all more reactive than in sections fixed in acid alcohol and similarly stained.

The method utilizing tetrazolium displayed distributions and intensities of staining reactions nearly identical with those when the specific reagent was utilized (fig. 10). The only obvious difference was that in some of the epithelial cells of epidermis, hair, and sebaceous glands, the diformazans were precipitated as fine crystals, whereas the reaction product of the specific reagent (DDD) was more homogeneous.

There was a good general agreement as to the sites of staining of mature, resting hair follicles in sections of skin of all species examined. Minor variations in the intensity of staining of the root sheaths were noted, and the outer third of the cortex in human hair contained occasional sulfhydryl-positive fibrils, whereas in the other animals this region contained only disulfides. However, variations in intensity of staining occurred in the stratum corneum and stratum granulosum of rats and other mammals. The reasons for this variability are not clear, but it is possible that there are minor differences in the amount and distribution of sulfhydryl and di-

sulfide in the epidermis of different species or in different parts of the epidermis of the same animal. Another reason for these differences may be due to a cyclic variation in sulfhydryl and disulfide of the epidermis (*30*).

Other stratified squamous epithelia.—The sulfhydryl content of the cells of the stratum Malpighii of the buccal mucous membrane was very similar to that of the adjacent epidermis at the mucocutaneous junction. An intense reaction for sulfhydryl occurred in the stratum granulosum of the mucous membrane but none in the epidermis. The stratum corneum of the former region reacted more intensely than that of the epidermis.

In the sections stained for both sulfhydryl and disulfide there was an intensification of the reaction in the two outer layers of the buccal mucous membrane. The color was slightly more intense than in the outer layers of the adjacent epidermis (fig. 21).

The histochemical reactions of the esophageal epithelium were similar to those of the buccal mucous membrane (fig. 23). Here there was also an intensified sulfhydryl reaction in the stratum granulosum. With the sulfhydryl and disulfide method both the stratum corneum and stratum granulosum were equally intensely colored.

In the stratified squamous epithelium of the cornea of both rat and monkey (fig. 19) disulfide groups appeared to be localized only in the outer, cornified cells. The same was true of the vaginal epithelium of rats in estrus (fig. 20).

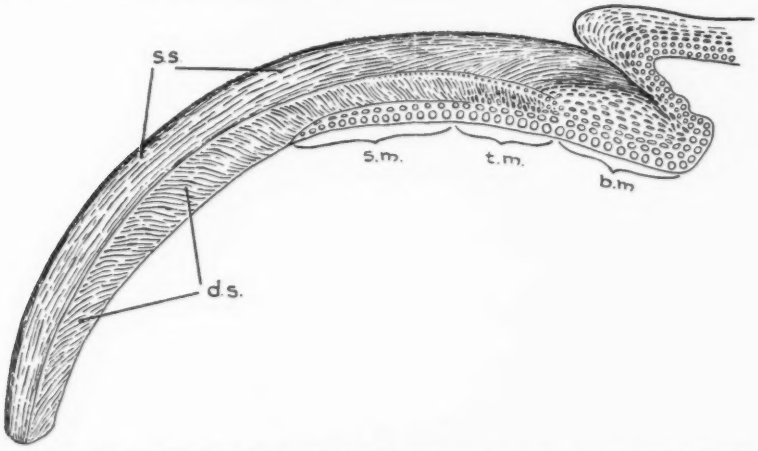
Chicken feather.—Plucked contour feathers of the white Leghorn chicken were studied in cross sections through three areas: through the quill or calamus that lies in the dermal follicle, and through the proximal and distal parts of the shaft or rachis of the vane that forms the exposed part of the feather. It was not possible to retain the barbs and barbules in the majority of sections through the vane.

The distal portion of the quill, especially the convex dorsal wall and portions of the lateral walls, was strongly reactive for sulfhydryl. The concave ventral side of the quill and the adjacent parts of the lateral walls were much less reactive. At the junction of the ventral and the lateral wall there was a clear transition zone from a moderate to an intense reaction in the lateral wall. The enclosed pith within the quill was negligibly reactive for sulfhydryl. The layers of the feather sheath which surround the quill were weakly to moderately stained. The walls of the quill were also intensely reactive for disulfide, excepting the ventral surface which contained little or none. The pith was strongly reactive for disulfide and the layers of the feather sheath were less reactive.

The walls of the proximal part of the shaft were positive for sulfhydryl, especially the ventral parts of the lateral walls (fig. 4). The reaction in the shaft was somewhat less intense than in the quill and no transition zone was noted between the ventral wall and the lateral walls. All four walls of this region of the shaft were intensely reactive for disulfide (fig. 5). The walls of the distal part of the shaft were weakly reactive for sulfhydryl, but the reaction for disulfide was much more intense. However, neither of the reactions was as intense as in the two other regions of the

feather. The pith of the shaft reacted similarly to that in the quill. Barbs and barbules, whenever retained in the sections, were positive for both sulfhydryl and disulfide.

Rat claw.—The present observations are in agreement with those of LeGros Clark (*31*), which divide the typical mammalian claw into superficial and deep strata and the epithelial claw matrix into basal, terminal, and sterile parts (*cf.* text-fig. 1). The most proximal or basal part of the matrix lies under the eponychium and consists of stratified squamous epithelium. There is a gradual transition between the flattened superficial cells of this basal matrix and the superficial stratum of the claw, the fibrils of which run horizontally. The distal or terminal part of the matrix is continuous in front and deep to the basal part. The stratified cells of the terminal part give rise to the fibrils of the deep stratum of the claw which are directed obliquely upward and forward toward the tip. In front of the basal and terminal portions of the matrix, together designated the germinal matrix, lies the sterile matrix, over the surface of which the claw glides during its continuous growth (figs. 7, 8).



TEXT-FIGURE 1.—Drawing of a midsagittal section of the claw of the rat, indicating the various regions. Modified from drawing by LeGros Clark (*31*). *s.s.*: superficial stratum; *d.s.*: deep stratum; *b.m.*: basal matrix; *t.m.*: terminal matrix; *s.m.*: sterile matrix.

The epithelium of each of the matrices and the fibrils nearest the basal and terminal matrices were moderately reactive for sulfhydryl, but appeared to contain no disulfide (figs. 7, 8). Distal to this region, in both strata of the claw proper, some fibrils were intensely reactive for sulfhydryl, especially in the proximal part of the superficial stratum. The rest of the fibrils of both strata were unreactive (fig. 7). In some instances, fibrils of the deep strata appeared to be arising from the surface of the sterile matrix, but there was no zone of sulfhydryl-positive fibrils present in this area as in the areas nearest the other matrices. Since most of the fibrils were unreactive in this region, and a transition

zone was not apparent, the present material is in agreement with the suggestion that the sterile matrix plays no role in the development of the claw (*31*).

With the method for combined sulfhydryl and disulfide, only the fibrils of the body of the claw were very intensely reactive, especially the fibrils of the superficial stratum (fig. 8).

Lamprey teeth.[6]—The horny teeth of the lamprey are interesting not only because they have been regarded as representing an early stage in the evolution of the teeth of vertebrates but also because they are composed of cornified or keratinized epithelium (*32*). The arrangement of the teeth in histologic sections is indicated in figures 1 and 2. The horny outer, or functional, cone-shaped tooth protrudes from the epidermis of the mouth and is periodically shed. Beneath this functional tooth is a vertical series of one or two developing replacement teeth, with a germinating tooth bud at the bottom. The teeth are separated by stellate reticulum except at the lateral margins where epidermal cells intervene.

Sections of lamprey teeth showed a variable reaction for sulfhydryl and for disulfide depending upon the stage of development of each tooth. The following are the major trends in the staining reaction for sulfhydryl: the outer, functional tooth displayed a negative or weak sulfhydryl reaction. The first replacement tooth reacted primarily at the apex and at the base of the conical tooth, while the subjacent developing tooth bud reacted strongly (fig. 1). The second replacement tooth, when present, also was strong in sulfhydryl. The reaction for disulfide was the reverse of that for sulfhydryl. The tooth bud was negative for disulfide, whereas the replacement teeth showed a successive increase in reaction which was maximal in the outer functional tooth (fig. 2). The epidermis surrounding the columns of teeth showed little reaction in comparison.

Nervous system.—The alcohol-insoluble protein components of the myelin sheaths of the sciatic nerve fibers, as well as other peripheral and central nervous system fibers, were unreactive for sulfhydryl, but reacted moderately for disulfide. The axons, on the other hand, were positive for sulfhydryl but showed little if any disulfide (fig. 26). The pattern of the staining reaction of the myelin sheaths was somewhat reminiscent of the reticulated appearance produced by the Ashbel-Seligman carbonyl reagent (*26*) and by Weigert's method (*12*).

The cytoplasm of some of the cells in the nervous system, such as the ventral horn cells of the spinal cord and those of the somatic motor nuclei of the brain stem, contained disulfide. The nuclear membranes, the nucleoli, and occasional granules in the nuclei of these cells appeared more prominent when stained for sulfhydryl and disulfide than for sulfhydryl alone. However, the nuclear structure contained fewer of these groups than the cytoplasm. Other cellular elements of the central nervous system contained fewer disulfides and some appeared to contain none. In the cerebellum, the fibers of the molecular layer and the cells of the

[6] This material was provided through the courtesy of Dr. Reidar F. Sognnaes, Harvard University School of Dental Medicine.

granular layer contained disulfides. The Purkinje cells showed a striking enhancement in the staining reaction for both sulfhydryl and disulfide over the sulfhydryl reaction alone, especially in the nuclear membrane and the perinuclear cytoplasm. In sections of cerebellum fixed in Zenker's fluid, some of the cells of the granular layer were more reactive than others, whereas they all reacted with equal intensity in sections fixed in acid alcohol. The lining of the ventricles and the chorioid plexus also revealed few disulfide groups.

Digestive system.—In the *sublingual gland*, the reaction of the basilar portion of the cytoplasm of the acinar cells was moderate for sulfhydryl, but the mucus was unreactive. The entire cytoplasm of the epithelial cells of the ducts was more reactive than that of the acinar cells. Sections stained for both sulfhydryl and disulfide revealed a greater intensity of reaction in both the acini and the duct epithelium (fig. 33), and there were no obvious differences between the sites of staining by the two methods.

The cytoplasm of the acinar cells of the *submaxillary gland* was weakly to moderately reactive; the basilar part of the cytoplasm usually being more reactive than the apical part. However, the tubular segment of the duct system, which lies between the acini and the excretory ducts as described by Lacassagne (*33*), was more strongly reactive for sulfhydryl than the acini. Staining occurred in rodlike or granular structures in the basilar portion of these cells but the apexes sometimes reacted strongly also. The entire cytoplasm of the excretory duct epithelium showed the strongest reaction of any part of the gland. The method for both sulfhydryl and disulfide increased the staining intensity somewhat in all parts, but mostly in the tubular portion of the duct system (fig. 34).

In the mucosa of the cardiac portion of the *stomach*, the distribution of sulfhydryl and disulfide groups was similar to that in the esophagus. In the fundic portion of the stomach, the cytoplasm of the mucous neck cells and of the parietal cells was slightly more reactive for sulfhydryl alone than that of the zymogen cells. The reaction in the cytoplasm of the mucous neck cells was homogeneous, while that of the parietal cells was somewhat granular. In sections stained for both sulfhydryl and disulfide, the reaction appeared somewhat intensified in all the cell types. However, after fixation in Zenker's fluid or other protein precipitants, the histochemical reaction was markedly intensified in the parietal cells in comparison to the others (fig. 6).

In the *duodenum*, the reaction for sulfhydryl and disulfide was more intense than the reaction for sulfhydryl alone, especially in the cuticular border of the columnar epithelium covering the villi and lining crypts of Lieberkühn. The mucus of the goblet cells was unreactive but the autonomic ganglion cells of the myenteric plexuses and the smooth muscle fibers reacted moderately.

In spreads of *mesentery*, the granules of the mast cells were moderately reactive for disulfide (fig. 24), as were thin, single elastic fibers.

Disulfides were present in *hepatic* cells in the same distribution as

sulfhydryl groups, involving the cell borders and granular structures in the cytoplasm as well as the nucleus. The over-all effect was merely an intensification of the reaction for sulfhydryl and disulfide over that for sulfhydryl alone.

The acinar cells of the *pancreas* fixed in acid alcohol contained few disulfide groups (fig. 27) and there were no essential differences in the pattern of staining by the two methods. Islet cells showed little reaction in comparison and no differences were noted in the intensity of staining of the different cell types (fig. 30). However, when the pancreas was fixed in Zenker's fluid or other fixatives that precipitate proteins, a different pattern was obtained. Although the basilar and perinuclear portions of the acinar cells were stained as before, the granules in the apexes reacted very intensely (fig. 28). The islet cells were also more intensely colored, but there were still no consistent and clear-cut differences in the staining of the various cell types (fig. 31).

Trachea.—The cilia and the cuticular border of the tracheal epithelium were more reactive for sulfhydryl and disulfide than for sulfhydryl alone (figs. 22, 32). Disulfide-reactive globules were present in the outer halves of the ciliated cells; these were smaller and more intensely reactive in the supranuclear region of the cell, and larger and less intensely reactive in the apexes (fig. 32). The globules were neither periodic-acid-Schiff-reactive nor metachromatic with toluidine blue. The clearly distinguishable mucus of the few goblet cells appeared unreactive. However, the region of the cytoplasm between the mucous goblet and the nucleus frequently contained disulfide. Elastic-tissue fibers (fig. 32) of the lamina propria reacted strongly for disulfide, whereas various stromal cells reacted feebly. The cytoplasm and secretory granules of the epithelium of the tracheal glands were positive (fig. 22).

Both the cells and fibers of the perichondrium and the chondrocytes of tracheal cartilage contained disulfide, but the ground substance was negative. At the open ends of the tracheal cartilage rings, where hyaline cartilage is replaced by elastic cartilage, the elastic fibers of the matrix were strongly reactive (fig. 22).

Kidney.—The glomeruli and all of the tubular elements of the cortex were more reactive for sulfhydryl and disulfide than for sulfhydryl alone. In sections fixed in Zenker's fluid the epithelium of the proximal convoluted tubules was reactive in both cytoplasm and brush border or in the brush border alone (fig. 12). The latter reaction was noted in those parts of the proximal tubules nearest the medulla. The cytoplasm of the cells of the distal convoluted tubules presented a uniform reaction. In the collecting tubules only the basilar portion of the cytoplasm appeared stained.

In the outer zone of the medulla, the epithelium of the straight portions of the proximal convoluted tubules and of the ascending limbs of Henle showed a moderate cytoplasmic reaction. The epithelium of the thin segments of the ascending limbs of Henle, the bends of the shorter loops, and the collecting tubules were, on the other hand, essentially unreactive.

In the inner zone of the medulla, the epithelium of the loops of Henle and of the collecting tubules was also negative.

Endocrine organs.—In sections of *adenohypophysis* fixed in acid alcohol, the reaction for sulfhydryl and disulfide was somewhat more intense than that for sulfhydryl alone. No clear-cut differentiation of cell types could be made on the basis of staining. However, after fixation in Zenker's fluid or other fixatives cited under Methods, the cytoplasm of the acidophils was stained more intensely than that of the other cell types (fig. 9). The cell types stained were determined by comparison of 3 μ serial sections stained by the sulfhydryl and disulfide method and by a selective trichrome method (*34*). The further characterization of these cells in various physiological states will be dealt with in another communication (*29*). The colloid material present in the residual cleft of the pituitary between the anterior and intermediate lobes was intensely reactive for disulfide groups, regardless of fixation, whereas it reacted only sparingly for sulfhydryl. The cells of the intermediate lobe appeared to be weakly reactive.

Sections of *neurohypophysis* fixed in acid alcohol showed no change in the intensity or distribution of staining for sulfhydryl and disulfide over that of sulfhydryl alone. However, sections of neurohypophysis fixed in Zenker's fluid or other fixatives gave a markedly different result. In these instances, the neurosecretory material was preserved and was found to be moderately stained for disulfide groups. This reactive material appeared as homogeneous, extracellular, colloidlike droplets or globules which varied widely in size (fig. 25) and seemed to be located around the capillaries. The cytoplasm of the pituicytes appeared to be unreactive. The nerve fibers of the neurohypophysis were stained after the manner of axons in general, as described in a previous passage. As a consequence, no decision could be reached as to whether any part of this reaction in the axons could be attributed to neurosecretory material.

In the *thyroid gland*, the follicular epithelium was somewhat more reactive for sulfhydryl and disulfide than for sulfhydryl alone, and there were moderately reactive granules in the apical portions of most of these cells. The colloid within the follicles contained some sulfhydryl and considerable disulfide groups and reacted with much greater intensity than the follicular cells (fig. 29).

The cytoplasm of the cells of the *parathyroid gland* of both the rat and the ox were weakly reactive for sulfhydryl alone and only slightly more intensely stained for both sulfhydryl and disulfide. There were no obvious differences in the intensity of staining in any of the cells of these two species.

The cells of the *adrenal cortex* contained a little disulfide. Contrary to the reaction for sulfhydryl, in which there was no difference in the intensity of staining of the cells of the various zones, the epithelium of the reticular zone was somewhat more reactive than that in the outer two zones. The cytoplasm of the cells of the medulla which was virtually unreactive for sulfhydryl showed a sparse reaction for disulfide. The

cytoplasm of adipose cells of the periadrenal fat also contained little disulfide.

Both the epithelium of the tubules of the *testes* and the interstitial tissue between the tubules contained disulfide groups, the latter showing a slightly more intense reaction than the former. In the seminiferous epithelium, the spermatids appeared slightly more reactive for disulfide than did the other spermatogenic cells. Spermatozoa reacted very intensely for sulfhydryl and it was difficult to estimate whether the intensity was increased by the sulfhydryl-disulfide reaction.

In the *thymus* of the rat, dog, cat, and monkey, all of the closely packed thymocytes reacted uniformly but weakly for either sulfhydryl or disulfide. Hassall's corpuscles, which gave a strong reaction for sulfhydryl, showed only little intensification with the sulfhydryl-disulfide method (fig. 17). The reaction appeared to be localized in the concentrically arranged "hyalinized" cells, and in the "degenerating" cells at the center of the corpuscles.

Blood vessels.—The endothelium of capillaries showed a stronger reaction for sulfhydryl and disulfide groups than for sulfhydryl alone, the latter being a very weak reaction. The elastic tissue of medium-sized (fig. 29) and large arteries (fig. 3) as well as of other tissues (figs. 22, 24) was reactive with the sulfhydryl-disulfide method as indicated on an earlier page; an intense reaction occurred after sulfide reduction, and a less intense reaction after thioglycolate or BAL reduction. Elastic tissue, however, failed to stain with the neotetrazolium method, or with the specific substrate after the disulfide groups had been split by cyanide or hydrosulfite. The histochemical reaction of elastic tissue which had been subjected to sulfide, thioglycolate, or BAL reduction was inhibited by iodine oxidation, or reaction with iodoacetate or N-ethyl maleimide prior to histochemical staining. Part of the increased staining reaction after sulfide reduction was due to the addition of the sulfide to reactive groups such as carbonyl, to produce a new fixed sulfhydryl. The specific reaction in elastic tissue, however, may be attributable to components other than elastin since elemental analyses have shown this protein to be virtually free of sulfur (*35*). The possibility nevertheless exists that elastin contains disulfides that may have been destroyed during its isolation. Elastic tissue is frequently boiled in strong alkali for long periods of time (*35*), a procedure that destroys disulfides (*13*).

Erythrocytes in the lumina of blood vessels reacted variously. After acid-alcohol fixation, they were usually unreactive for either sulfhydryl or disulfide. After fixation in Zenker's fluid or other protein precipitants, they were usually well stained. After formalin fixation and thorough washing, the erythrocytes were positive for sulfhydryl (hemoglobin) and for sulfhydryl and disulfide.

Discussion

The experiments presented herein indicate that the reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD), may be applied in satisfac-

tory, specific methods for the histochemical demonstration of sulfhydryl and disulfide groups of proteinaceous material. Various reducing agents were able to convert the disulfide groups to sulfhydryl for reaction with DDD. Some loss of specificity was encountered only with ammonium sulfide and other inorganic sulfides as reducing agents. Since these sulfides were particularly good reducing agents, in order to use them we went to some trouble to develop methods that depended on blocking the carbonyl groups responsible for artefacts. However, a simpler method for demonstrating sulfhydryl and disulfide groups was devised with thioglycolate as the reducing agent. This method did not give as accurate an estimation of the amount of these groups as the published method for sulfhydryl groups (*2*, *3*), because of differences in availability for reduction and in rate of reduction of disulfides of various proteins (*9*, *19*, *36*), and the unknown and incomplete degree of reduction under the conditions selected. When more drastic conditions were employed for carrying out a more complete reduction, morphologic details were sacrificed. In order to estimate disulfide content, it was necessary to compare slides stained for sulfhydryl with those stained for both. This type of study is usually unsatisfactory, as it requires particularly careful observation and subtle differences go unobserved or at least cannot be given significance with confidence.

A method for demonstrating only disulfide required blocking sulfhydryl groups irreversibly before reducing the disulfide. Although several sulfhydryl-blocking agents were used, none of these withstood strong reducing agents so that the weak splitter, potassium cyanide, had to be used to open the disulfide. A control slide to show that sulfhydryls were successfully blocked was always prepared.

In all these methods, as previously pointed out for sulfhydryl (*2*), the color of the pigment gives some idea of the number and proximity of reactive groups due to the fact that monocoupling with tetrazotized diorthoanisidine yields a red color, whereas dicoupling results in a blue color.

The method based on the oxidative properties of ditetrazolium chloride (BT) lacked specificity, and required control slides and a high *p*H that was destructive of all but tough fibrous proteins such as the keratins of skin.

Only a beginning was made, in the present study, of the effect of various fixatives on the subsequent demonstration of sulfur-containing groups of protein. It was clearly shown that many proteins, which are soluble in trichloroacetic acid–alcohol, may be rendered insoluble enough for histochemical demonstration if other fixatives are used. Some of these proteins are particularly interesting because they are hormones or excretory enzymes. The method apparently did not preclude the use of mercaptide-forming fixatives, because these metals were reversibly removed under the conditions employed (especially with ammonium sulfide or sulfhydryl-containing reductant). It should be emphasized, however, that it was not possible to indicate, on the basis of histochemical

staining alone, whether the staining was due to sulfhydryls, disulfides, or both, when fixatives which are oxidants or mercaptide-formers were used.

A few examples illustrate this point. After fixation in Zenker's fluid, the cellular protein of the proximal convoluted tubules of the kidney, the parietal cells of the stomach, the apical portion of tracheal epithelial cells, and of erythrocytes was stained for sulfhydryl and disulfide, but it was not possible to tell which, if either, of the groups predominated. It is certainly worth noting and it may be significant that carbonic anhydrase, which is found in the parietal cells of the stomach and renal cortex and in erythrocytes, is soluble in alcohol and contains appreciable disulfide (*37*). However, in the case of the staining of the neurosecretory material of the neurohypophysis, the reaction was attributed to disulfide only, for the following reasons: The reaction in the neurohypophysis disappeared upon prior dehydration of the animals and upon stress, and reappeared upon hydration of the animals. The reaction was also affected by section of the stalk of the pituitary gland (*27*). This was strong presumptive evidence that the stained material in the neurohypophysis was related to antidiuretic hormone. Analysis of samples of this protein hormone revealed moderate amounts of disulfide and no sulfhydryl (*38*, *39*). Furthermore, this protein reacts positively *in vitro* by the present method for disulfide, and negatively for sulfhydryl (*27*).

By similar experiment and argument the demonstration of disulfides in the apical portion of pancreatic acinar cells fixed in Zenker's fluid was attributed to the digestive enzymes, amylase and chymotrypsinogen (*9*, *39*), and possibly other peptidases (*40*) in these sites. By using still another fixative, insulin has been demonstrated in islet cells of the pancreas (*28*). It would appear that the sites of a variety of proteins containing sulfhydryl or disulfide groups can be demonstrated by the selection of an appropriate fixative and by correlation of the histochemical results with *in vitro* analysis of the pure protein and physiological experiments combined with histochemical study of the tissue.

Although glutathione was not demonstrable after fixation of tissue in acid alcohol, it was not certain whether it would be demonstrable after fixation in Zenker's fluid in which it was rendered insoluble in *in vitro* experiments. Since reduction of disulfide would be required after the use of oxidative fixatives, it was possible that glutathione might be eliminated from the sections during this procedure. The development of a method for glutathione is under investigation, utilizing acylating fixatives that do not oxidize or combine with the sulfhydryl of glutathione but increase its molecular weight and decrease its solubility (*10*).

Other methods have been used to demonstrate disulfides. The nitroprusside reaction (*41*, *42*) or the ferricyanide reaction (*43*) have been used after thioglycolate reduction (*24*, *21*). However, these methods lack specificity (*3*). Danielli (*44*) suggested a method based on blocking sulfhydryls with phenylisocyanate and cleavage of disulfides with cyanide. Dinitrofluorobenzene was used to combine with the sulfhydryls so pro-

duced, and this was followed by reduction of nitro to amino, diazotization and coupling. However, this ingenious method has not given satisfactory results (*45*). Three other methods of questionable practical use, reliability, and specificity are said to depend on the oxidation of cystine, to cysteic or sulfonic acids for example, and staining of the products thereby produced (*46*, *47*, *48*).

In the brief survey of the histochemical reactions for disulfide in the tissues of rats and other animals, it was shown that compounds, presumably protein in nature, which contain these groups are frequently localized in many glandular and surface epithelial cells, nerve cells, muscle fibers, and connective-tissue cells that also contain sulfhydryl groups. In these tissues, the total result of the staining reaction for sulfhydryl and disulfide was merely intensification over that for sulfhydryl alone. This superimposition of the two is not unexpected since sulfhydryls and disulfides provide an intracellular oxidation-reduction system in many tissues (*9*, *11*). The reversibility of the system, $2\,SH - 2e^- \leftrightarrows S\text{–}S + 2H^+$, and the specificity of the reactants (SH and S–S) for each other (*11*), suggest that in many instances they would be found at the same sites. However, frequently a predominance of either sulfhydryl or disulfide groups was found in cells, indicating that the equilibrium was shifted far to one side or the other. A number of protein hormones contain disulfides that are important for their biological activity. This has been amply demonstrated in the case of insulin [see reviews (*9*, *49*)], and also for posterior pituitary hormones (*38*, 50), and many of the protein hormones of the anterior pituitary gland (*9*, *51*, *52*), although there is still some controversy concerning the relationships of disulfides to activity of the hormones of the pituitary gland. Parathormone also contains disulfide groups (*53*) as does pregnant mare serum (PMS) (*54*). Nevertheless, many cells of the parathyroid gland of the rat and of the ox failed to give a significantly intense reaction for sulfhydryl or disulfide. Digestive enzymes contain appreciable quantities of disulfides (*9*, *40*) as do many other enzymes (*9*, *11*). The rather widespread occurrence of disulfide in proteins, other than enzymes and hormones, is reviewed elsewhere (*9*, *11*, *55*, *56*).

Of particular interest were the sites that contained few or no sulfhydryl groups after acid-alcohol fixation, but contained disulfide after this fixation, or both sulfhydryl and disulfide after special fixation. These areas included the distal half of hair shafts, the strata of claws, the outer horny teeth of the lamprey eel, pancreatic acinar cells, and pancreatic islet cells, mast cell granules, tracheal epithelium, the brush border of proximal convoluted tubular epithelium of the kidney, some acidophils of the adenohypophysis, neurosecretory material of the neurohypophysis, colloid material of the residual cleft of the pituitary gland, the alcohol-insoluble residue of myelin sheaths of nerve fibers, erythrocytes, and elastic tissue. Tissue such as the stratum corneum of various stratified squamous epithelia, the quill and shaft of chicken feathers, and Hassall's corpuscles are listed separately because, although they contained disulfides, they also contained appreciable amounts of sulfhydryl. The significance of some of these disulfide-

containing proteins has been presented above. Areas in which negligible or no disulfide was demonstrated were axoplasm of nerve axons, the medulla and the bulb of hair, the basal cells of epidermis and other stratified epithelia, collagenous tissue, and the ground substance of cartilage.

The keratins are another class of disulfide-containing proteins, which probably contribute to the architecture of certain epithelial cells by virtue of their fibrous nature. Of the many proteins designated as keratins, the eukeratins (hard keratins) yield, on acid hydrolysis, histidine, lysine, and arginine in the approximate ratio of 1:4:12 (*57*), whereas the pseudokeratins (soft keratins) yield these amino acids in the approximate ratio of 1:3:3 (*21*). The cystine content, on the other hand, varies widely even in the eukeratins. There is, for example, about 26 times more cystine in human hair than in human dental enamel (*57*). Less is known about the cystine content of the pseudokeratins.

It has been suggested that epidermal structures contain two types of structural protein in varying proportions, a fibrous protein with low sulfur content and a nonfibrous protein with relatively high sulfur content (*21*). This suggestion may explain our histochemical observations demonstrating varying proportions of disulfide and sulfhydryl in tissues that are known to contain the keratins. For example, by using both methods for sulfhydryl and for disulfide we have shown that hair, claws, and the epidermal lamprey tooth, are intensely reactive for sulfhydryl, but not for disulfide, in the Malpighian layer of the hair bulb, claw matrix, and tooth bud. The distribution of these groups in fully formed keratinized structures, *e.g.* outer portion of hair shaft, strata of the claw, and horny layer of lamprey tooth, is reversed. However, it should be noted that a sulfhydryl reaction was always obtainable in the keratinized portions of claws and lamprey teeth, but rarely occurred in the cortex of the outer half of hair. Moreover, claws and lamprey teeth resembled epidermis which contains a sulfhydryl-positive stratum Malpighii and a sulfhydryl- and disulfide-positive stratum corneum.

The present observations on the distribution of sulfhydryl and disulfide groups in epidermis, claw, and feather are not in agreement with those of Giroud and Bulliard (*42*, *58*). Neither is there agreement with the suggestion that free sulfhydryl groups disappear completely at the point of formation of either eukeratins or pseudokeratins from their sulfhydryl-containing precursors (*22*, *43*). Rather, in all keratinized tissues described above, the Malpighian zone contained only sulfhydryl, whereas the keratinized zone contained a variable amount of disulfide and some sulfhydryl as well.

The near absence of both groups does not support the concept that the medulla (*22*, *42*, *58*) and the inner root sheath (*59*) of hair contain keratin or that trichohyaline granules are keratin precursors (*59*), although confirmation is provided for the sites of sulfhydryl and disulfide in the hair cortex reported by others (*22*, *24*, *30*). The present data are, however, in agreement with the chemical analyses of hair (*57*, *60*).

By the present histochemical methods and by chemical methods (*57*),

the stratum corneum of the epidermis of rats and other mammals contains disulfides but much less than the hair cortex. However, the distinction between soft and hard keratin on the basis of an intensified sulfhydryl reaction in the area immediately below the keratinized zone in only the latter type (*22*, *42*) was not confirmed. Absence of staining of keratohyaline granules by either method does not confirm the impression that they are immediate precursors of keratin (*43*).

The presence of sulfhydryl in addition to disulfide in the keratinized portions of epithelium and epithelial derivatives is as yet unexplained. Also unexplained is the mechanism for the presumptive oxidation of sulfhydryls to disulfide during the process of keratinization. Other stratified squamous epithelia deserve investigation with these new techniques, *i.e.*, esophagus, cornea, vagina. The presence of sulfhydryl and disulfide groups and positive birefringence (*10*) in the stratum corneum and of only sulfhydryls in the stratum Malpighii of these structures is similar to findings in the epidermis, although the other epithelia are said not to contain keratin (*61*). Hassall's corpuscles in the thymus and the so-called hyperkeratinization of ordinarily nonkeratinized epithelia occurring in vitamin A deficiency also deserve further investigation.

Summary

The present paper deals with the application of a specific reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD), that was developed for the histochemical detection of protein-bound sulfhydryl groups, to the histochemical detection of sulfhydryl and disulfides simultaneously, or of disulfides alone. The use of tetrazolium salts to detect sulfhydryls and disulfides simultaneously, but less specifically, is also described. The influence of various fixatives on the histochemical reaction, the efficacy of various reducing agents in reducing disulfides, and the specificity of each of the three histochemical methods is given.

The distribution of disulfides in some of the tissues of albino rats and other animals is described. The significance of some of the sites of disulfides is compared with the results of other investigators and special reference is given to epidermal derivatives, especially the keratins.

The applicability of the present methods is discussed and examples are provided to indicate that the information gained by these histochemical methods may be supplemented with profit by information gained from *in vitro* analysis and physiological experiments to determine the topographical localization of certain specific proteins.

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PLATES

PLATE 52

Sections used for photomicrographs to indicate the presence of both sulfhydryl and disulfide groups were taken from blocks of tissue fixed in alcohol–trichloracetic acid and were reduced with thioglycolate before application of the specific reagent 2,2′-dihydroxy-6,6′-dinaphthyl-disulfide (DDD). These figures should be compared with the previously published photomicrographs of tissues stained for sulfhydryl only (2, 3). In some instances the sulfhydryl and disulfide method was used after fixation in Zenker's fluid, in others the disulfide method alone or the ditetrazolium chloride (BT) method after acid alcohol was used, and a few tissues were stained for sulfhydryl only.

FIGURE 1.—Lamprey tooth. *Sulfhydryl* reaction only. The tooth germ is strongly reactive for sulfhydryl, whereas the replacement tooth shows a weak and scattered reaction except at the base of the conical tooth. The outer, functional tooth is weakly reactive, but indicates primarily its natural color. × 35

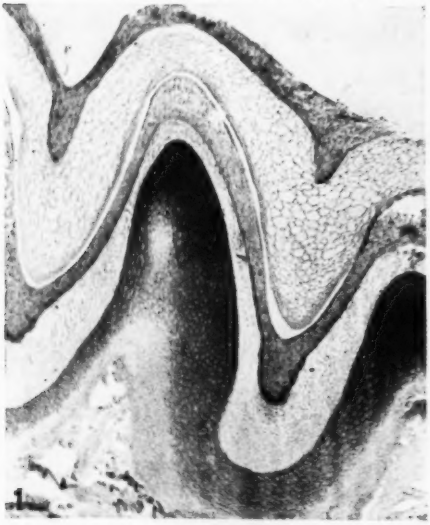
FIGURE 2.—Lamprey tooth. Ditetrazolium chloride (BT) method for *sulfhydryl and disulfide*. The outer functional tooth as well as the two replacement teeth are intensely reactive. Reaction of the inner tooth germ was considered due to sulfhydryl groups only, since there was no increase in the intensity of the reaction compared with sections stained for sulfhydryl alone. × 35

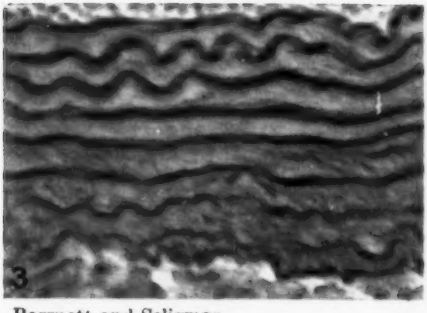
FIGURE 3.—Rat aorta. Elastic tissue stains moderately intensely for *sulfhydryl* after blocking of aldehydic carbonyl groups with semicarbazide and reduction with thioglycolate. The reaction for sulfhydryl alone was completely negative. Smooth muscle between the elastic lamellae is positive. × 200

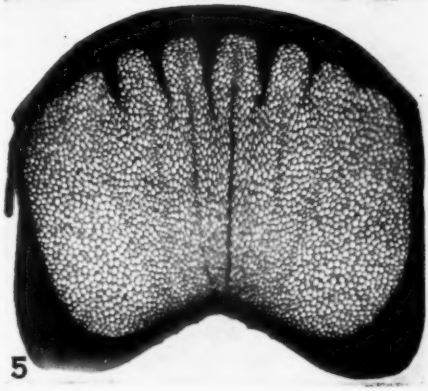
FIGURE 4.—Shaft of chicken feather. *Sulfhydryl* reaction only. Lateral walls of the shaft especially toward ventral surface (*bottom*) are intensely reactive for sulfhydryl. Other walls, the ventral and the dorsal, contain less and the central pith is very slightly reactive. × 37

FIGURE 5.—Shaft of chicken feather. Section parallel to figure 4, stained for sulfhydryl and disulfide. Note the increase in reaction due to *disulfide* in the walls of the shaft and in the pith. × 37

FIGURE 6.—Rat stomach. Section from block fixed in Zenker's fluid. Note the intense reaction for *sulfhydryl and disulfide* in parietal cells in comparison to the other epithelial-cell types. In acid-alcohol-fixed sections, no differentiation in staining of cell types was noted. × 100







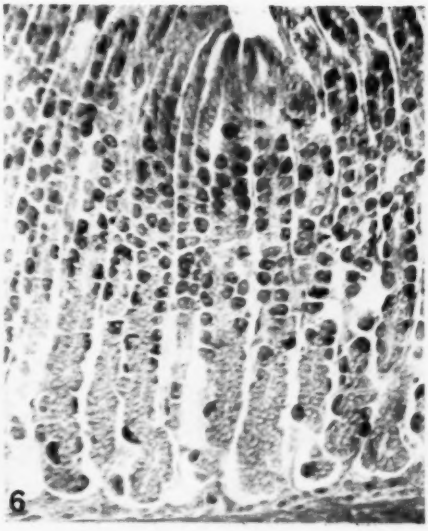


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PLATE 53

FIGURE 7.—Rat claw. *Sulfhydryl* reaction only. Sulfhydryl-positive fibrils are scattered throughout the superficial and deep strata of the claw. The sterile matrix and underlying tissue are also sulfhydryl-positive. × 37

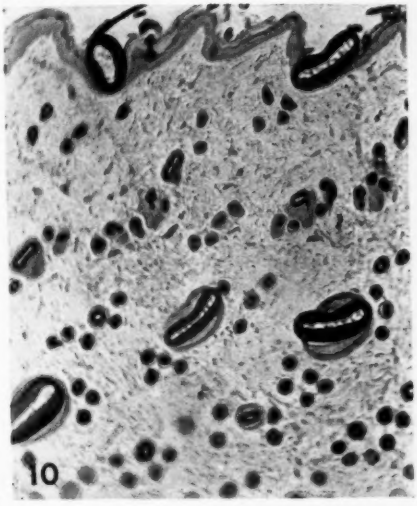
FIGURE 8.—Rat claw. *Sulfhydryl and disulfide* reaction. Note the increase in reaction of the fibrillary structures of the claw due to disulfides, especially in the superficial stratum. The matrix does not appear more reactive than in figure 7, indicating the absence of disulfides. × 37

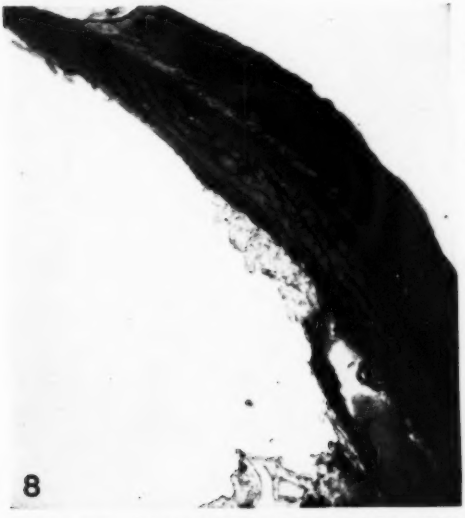
FIGURE 9.—Anterior lobe of pituitary gland of rat. After fixation in Zenker's fluid but not in acid alcohol, acidophils appear intensely reactive for *sulfhydryl and disulfide* in comparison to other cell types. × 450

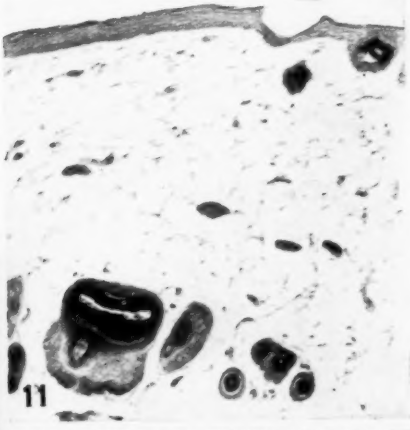
FIGURE 10.—Rat skin. Ditetrazolium chloride (BT) method. Note the intense reactivity of the cortex of the hairs, whereas the root sheaths are less intensely stained for *sulfhydryl and disulfide*. In the epidermis of this specimen, the stratum granulosum was more reactive than the other strata. × 50

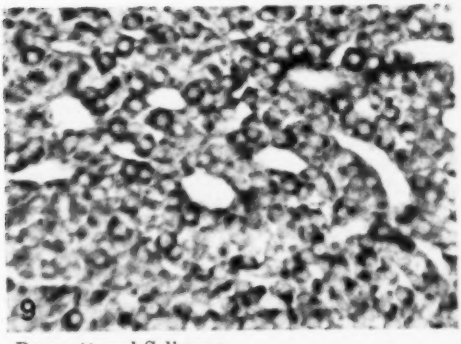
FIGURE 11.—Rat skin. The outer portion of the hair shafts, which are negative for *sulfhydryl*, react intensely for *sulfhydryl and disulfide*. In this specimen of epidermis the stratum granulosum is not intensely stained. × 90

FIGURE 12.—Cortex of rat kidney. Zenker's fluid fixation. The brush border is more reactive for *sulfhydryl and disulfide* than is the cytoplasm of the proximal convoluted tubules. In the other tubular elements the reaction is homogeneous. The reaction in the brush border is not present after acid-alcohol fixation. × 200









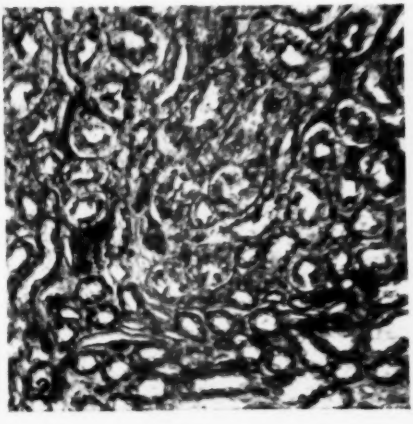


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PLATE 54

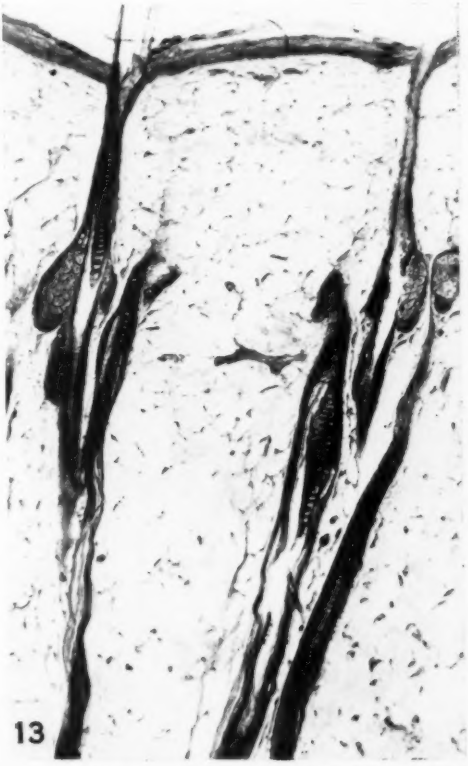
FIGURE 13.—Rat skin. *Sulfhydryl and disulfide* reaction. Longitudinal section through hairs showing the intense reaction in the hair cortex due to disulfide. In the epidermis, the stratum corneum shows a greater reaction than in sections stained for sulfhydryl alone, whereas the stratum Malpighii and sebaceous glands do not. Connective tissue shows little reaction. × 85

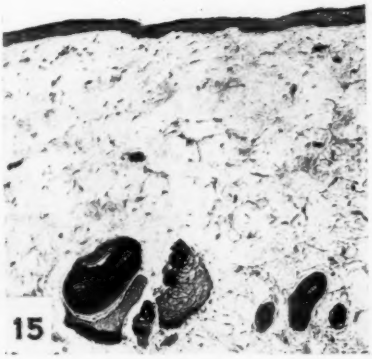
FIGURE 14.—Rat dermis. Section through base of hair follicles, a region which is intensely reactive for *sulfhydryl*, but not for disulfide. Compare with figure 16. × 100

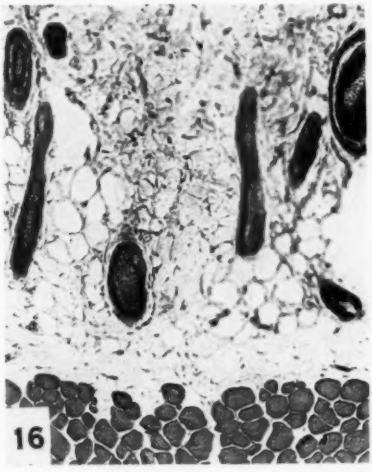
FIGURE 15.—Rat skin. *Disulfide* reaction only. In the epidermis only the stratum corneum appears reactive. The cortex of the hair is intensely stained, but not the sheaths or sebaceous glands. × 100

FIGURE 16.—Rat dermis. *Disulfide* reaction only. Section of area below that shown in figure 15. In comparison to figure 14, which shows a similar region positive for sulfhydryl, this section indicates the absence of disulfides in the bulb of the hair and a weak reaction in the hair cortex in the region of horny transformation. × 100

FIGURE 17.—Kitten thymus. Note the intense reaction for *sulfhydryl and disulfide* in the Hassall's corpuscles. The cells of the thymus are much less intensely stained. × 350







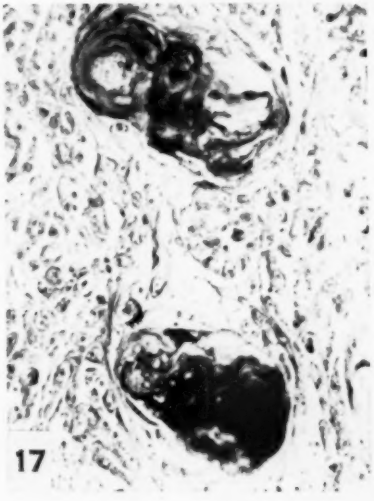


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PLATE 55

FIGURE 18.—Armadillo epidermis. Note the intense reaction for *sulfhydryl and disulfide* in the stratum granulosum. The stratum corneum contains most of the disulfide groups. × 100

FIGURE 19.—Monkey cornea. In comparison to sections stained for sulfhydryl alone, only the outer or cornified part of the cornea shows an intensified reaction for *sulfhydryl and disulfide.* × 350

FIGURE 20.—Rat vagina. *Disulfides* are present only in the outer cornified part of the estrous vaginal epithelium. × 350

FIGURE 21.—Rat lip. *Sulfhydryl and disulfide* reaction. The stratum granulosum of the mucous membrane of the lip is somewhat more reactive than that of the adjacent epidermis and is due primarily to sulfhydryls. The stratum corneum of the mucous membrane is also more reactive than that of the epidermis. A slightly more intense reaction in the stratum granulosum of the mucous membrane is noticeable. × 200

FIGURE 22.—Rat trachea. The epithelium is reactive for *sulfhydryl and disulfide,* especially the apical part, and in the lamina propria elastic fibers are intensely stained. In the midportion of the photomicrograph, the elastic fibers at the distal ends of the tracheal cartilage are reactive, but in the lower part of the photomicrograph the midportion of a cartilage ring shows no reaction in the matrix while the cells are positive. Striated muscle at the base is reactive. × 90

FIGURE 23.—Rat esophagus. The reaction for *sulfhydryl and disulfide* in esophageal epithelium is similar to that in the mucous membrane of the lip and the cardia of the stomach. Disulfide groups are present primarily in the stratum corneum. × 100

FIGURE 24.—Rat mesentery. Single elastic fibers and mast cells are reactive for *sulfhydryl and disulfide,* but not for sulfhydryl alone. × 300

FIGURE 25.—Rat neurohypothesis. The neurosecretory material is reactive for *sulfhydryl and disulfide* after fixation in Zenker's fluid; the reaction does not appear with acid alcohol. The positively stained material is depleted by dehydration of the rats. On the left, the cells of the intermediate lobe are weakly reactive. × 100

FIGURE 26.—Rat sciatic nerve. *Sulfhydryl and disulfide* reaction. The axons but not the myelin sheaths of peripheral and central nerve fibers are positive for sulfhydryl but the staining reaction is increased by the sulfhydryl and disulfide reaction only in the myelin. × 250

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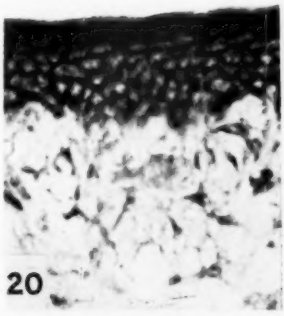
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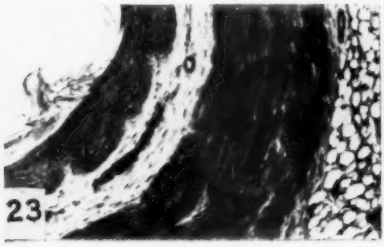
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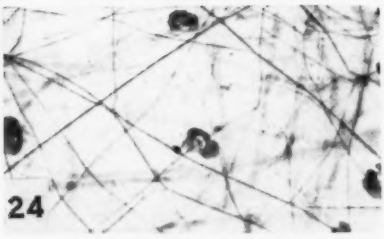
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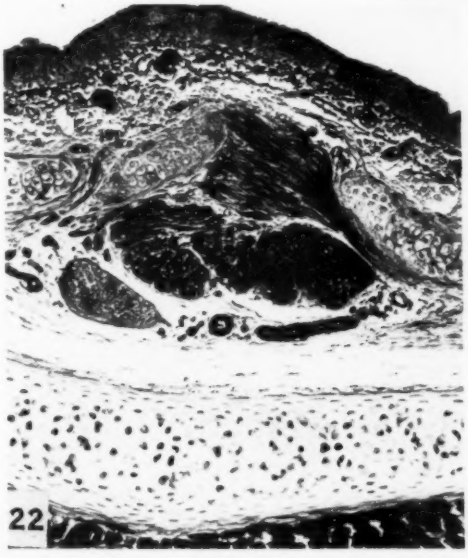
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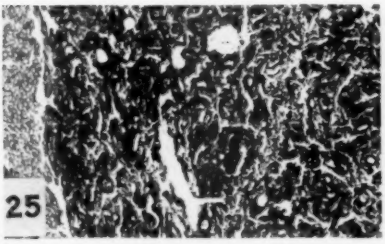
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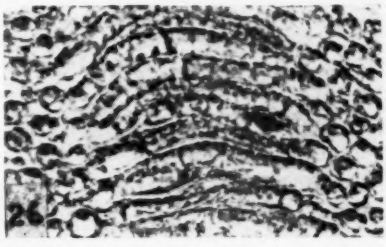
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PLATE 56

FIGURE 27.—Rat pancreas. The *sulfhydryl and disulfide* reaction is similar but more intense than the sulfhydryl reaction alone after acid-alcohol fixation. Staining is localized primarily to the apical and basilar parts of the acinar cells. × 250

FIGURE 28.—Rat pancreas. After fixation in Zenker's fluid, intensely reactive *sulfhydryl- and disulfide*-containing material is present in the apical part of the acinar cells. This material is depleted by pilocarpine injection. × 250

FIGURE 29.—Rat thyroid gland. The follicular colloid material is more reactive for *sulfhydryl and disulfide* than for sulfhydryl alone; the follicular epithelium less so. A medium-sized blood vessel is present to the right, showing a reaction in the internal elastic membrane. × 100.

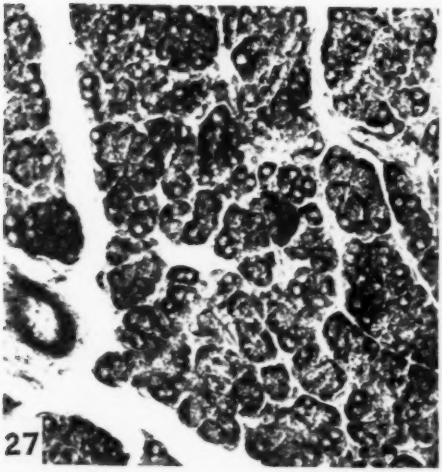
FIGURE 30.—Rat pancreatic islets. There is virtually no reaction for *sulfhydryl and disulfide* in the cells of the islet of Langerhans after acid-alcohol fixation. × 250

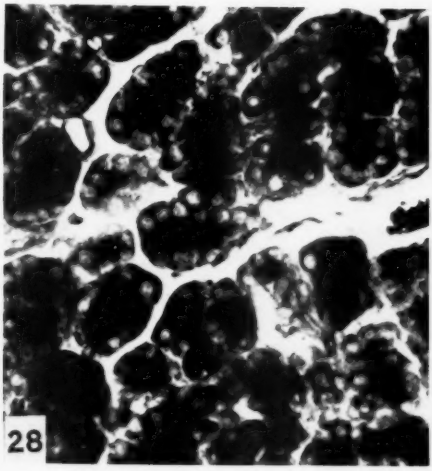
FIGURE 31.—Rat pancreatic islet. After fixation in Zenker's fluid, all the cells of the islet react with equal intensity for *sulfhydryl and disulfide*. × 250

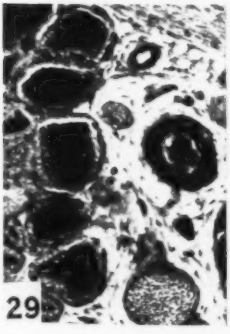
FIGURE 32.—Rat trachea. Enlarged photomicrograph of section of tracheal epithelium indicating the globular material in the apexes of the cells which is positive for *sulfhydryl and disulfide*. × 350

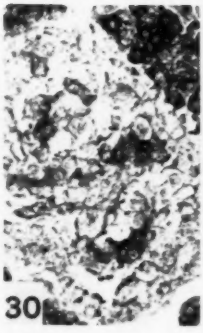
FIGURE 33.—Rat sublingual gland. The bases of the acinar cells are positive for *sulfhydryl and disulfide* but the mucus is negative. Excretory ducts show a stronger reaction. × 100

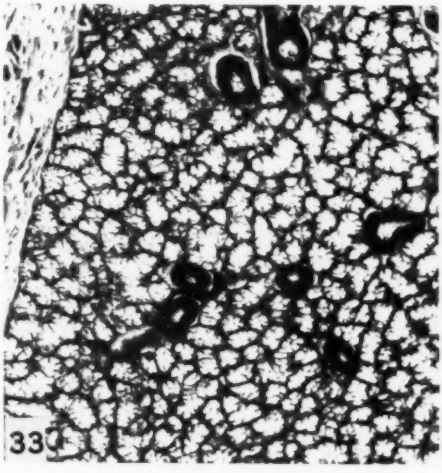
FIGURE 34.—Rat submaxillary gland. The acini are weakly reactive for *sulfhydryl and disulfide*, the tubular portion of the duct system more strongly reactive, and the excretory ducts show the most intense reaction. × 100

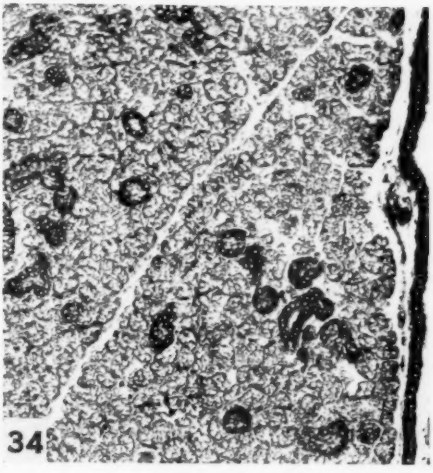












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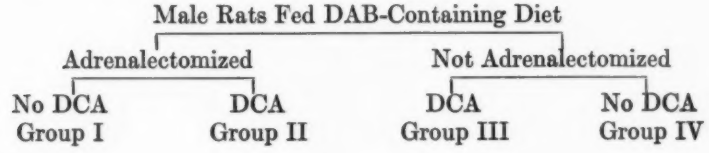
Effect of Adrenalectomy and of Desoxycorticosterone Acetate on the Formation of Liver Lesions in Rats Fed *p*-Dimethylaminoazobenzene [1]

ALEXANDER SYMEONIDIS,[2] AMBADAS S. MULAY, *and* FREDERICK H. BURGOYNE,[3] *National Cancer Institute,*[4] *Bethesda, Md.*

Experience with human and animal neoplasms has indicated that substances necessary for the normal functioning of the body may influence tumor development. Cancer of the human liver develops more frequently in males than in females (*1–3*). Experience with spontaneous and experimentally induced liver tumors in animals agrees with this observation of difference in response according to sex difference (*4–8*). Sex hormones significantly influence the development of liver tumors induced by carcinogenic compounds (*9, 10*). Taking into consideration the fact that liver regeneration in lobectomized rats is impaired by adrenalectomy and promoted by adrenal cortical hormones (*11–13*), an attempt was made in this study to determine whether or not adrenalectomy and/or administration of desoxycorticosterone acetate (DCA) would influence the development of liver tumors in rats fed *p*-dimethylaminoazobenzene (DAB).

Materials and Methods

Two hundred nineteen male Osborne-Mendel rats four to seven weeks old were divided into four groups. The rats were fed a semisynthetic diet containing *p*-dimethylaminoazobenzene (DAB). The animals in groups I and II were adrenalectomized and those in groups III and IV were not; the animals in groups II and III received desoxycorticosterone acetate (DCA), while those in groups I and IV did not. This treatment of the groups is graphically shown below:



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The composition and preparation of the semisynthetic diet used in these studies are described in detail by Mulay and Firminger (*14*). It consisted of 78 percent sugar, 12 percent casein, 4 percent salt mixture, 1 percent vitamin mixture, and 5 percent corn oil. *p*-Dimethylaminoazobenzene (DAB) was incorporated in the corn-oil fraction of the diet, in the amount of 0.06 gm. per 100 gm. of diet.

Adrenalectomy was performed in rats of groups I and II through a paravertebral incision under anesthesia produced by pentobarbital given intraperitoneally. The adrenal glands were removed with the adherent fat tissue, fixed in Zenker's acetic fluid, embedded in paraffin, stained with hematoxylin and eosin, and examined microscopically. In only two instances were the capsules of the adrenal glands found to have been ruptured during extirpation.

The DCA pellets were cylindrical and each consisted of 16 mg. of pure crystalline desoxycorticosterone acetate. They were implanted subcutaneously by trocar. The rats in groups II and III received a new set of four DCA pellets every four weeks, and previously implanted pellets were left in the animals. Each animal received a total of five sets of four pellets each during the duration of the experiment, a total of 320 mg. of DCA. The mean absorption of DCA per rat at the end of the experiment was 215 mg. in group II and 225 mg. in group III. The duration of the experiment was 189 days, from the day the rats were given the synthetic diet containing DAB.

Group I consisted of 112 rats adrenalectomized two weeks prior to being placed on the synthetic diet containing DAB.

Group II: 41 adrenalectomized rats received the DCA pellets subcutaneously. Two weeks after adrenalectomy the rats were placed on the synthetic diet containing DAB.

Group III: 27 nonadrenalectomized rats received DCA pellets subcutaneously and at the same time were placed on the synthetic diet containing DAB.

Group IV: 39 nonadrenalectomized rats were fed the synthetic diet containing DAB.

Two additional groups of rats were used as controls:

Group V: 21 nonadrenalectomized rats were fed the plain synthetic diet.

Group VI: 33 nonadrenalectomized rats were fed Derwood rat pellets. These pellets were composed of: hard wheat 65.1 percent, skim-milk powder 23.3 percent, dried brewer's yeast 4 percent, corn oil 5.8 percent, iodized sodium chloride 1.4 percent, ferric citrate 0.13 percent, shark oil 0.2 percent, and Delstrol 0.08 percent. The shark-oil and Delstrol fractions contained vitamins A and B in an amount equivalent to that in cod-liver oil at 2 percent of the diet (*15*).

Each rat was caged separately and its food intake was checked twice a week. No difference in the food consumption by the animals of the different groups was observed. All animals had free access to drinking water except those in group I, which received 0.9 percent sodium chloride. All

rats that survived for 189 days (on the carcinogenic diet) were sacrificed by ether and autopsied.

At autopsy the organs were carefully examined with the dissecting microscope. Special attention was paid to the adrenal glands and to the liver for tumors, and any liver lesion discovered was removed for microscopic examination. At least ten sections from each liver were examined histologically. The perirenal fat in the adrenalectomized animals was carefully examined for adrenal tissue. The nonabsorbed remnants of the implanted DCA pellets were dissected from the subcutaneous tissue, desiccated, and weighed. Specimens for histologic study were fixed in Zenker's acetic fluid, embedded in paraffin, and stained with hematoxylin and eosin and van Gieson's stain, and treated by the periodic acid-Schiff technique and the Wilder method for reticulum. Formalin-fixed material was used in staining for lipoid substances.

Results

Liver changes.—Liver tumors and early neoplastic liver lesions found in these animals have been classified for the evaluation of the results of this experiment into three categories:

A) Hepatomas (H). In this group have been included all fully developed tumors of the liver parenchyma; namely, liver-cell and bile-duct neoplasms (fig. 1). All forms of liver tumors like those described by Edwards and White (hepatoma I and II and adenocarcinoma) (*16*) and by Opie (trabecular hepatoma, adenohepatoma, cystadenoma, cholangioma) (*17*), were present in this group.

B) Atypical liver-cell areas (ALC), considered as early neoplasias (*16*), were characterized by basophilia of the regenerating liver cells, anisocytosis, increased prominence of the nucleoli, and a very large nucleus in some cells. A beginning or progressive destruction of the ctyoarchitectural form of the lobule could be seen in these areas (fig. 2).

C) Atypical newly formed bile ducts (ABD). Such areas consisted of extensively proliferating, hyperplastic bile ducts with cuboidal lightly stained epithelium, which in places became columnar with basophilic protoplasm containing a deeply stained and enlarged nucleus, prominent nucleolus, and mitosis. A rich stroma of cellular connective tissue surrounded these atypical bile ducts (fig. 3). These formations correspond microscopically to those described as "bile duct adenomatosis" [Sugiura and Rhoads (*18*)], or "cholangiofibrosis" [Opie (*17*)]. With the exception of Edwards and White (*16*), the majority of authors who have studied this lesion considered cholangiofibrosis as an early neoplastic lesion (*17–20*).

Combinations of all of these lesions were present in the livers of rats with fully developed tumors. Atypical bile-duct formations were found in livers with atypical liver-cell areas. All these neoplastic liver formations combined will be designated as neoplastic liver lesions in this study.

In group I (adrenalectomized rats fed DAB), 12 of 29 rats surviving for 189 days (41.4 percent) had neoplastic lesions of the liver: 2 rats were found with fully developed tumors of the liver parenchyma (H); 2 animals

had atypical liver-cell areas (ALC); and 8 rats had atypical bile-duct formations (ABD). Every one of these rats had regenerating adrenal cortical nodules.

In group II (adrenalectomized rats fed DAB and treated with DCA), none of the rats developed hepatomas. Two rats had areas of atypical liver cells (ALC), and one rat had atypical bile-duct formation (ABD). These three rats had regenerating adrenal cortical tissue.

In group III (intact rats fed DAB and treated with DCA), the livers of all animals were free of neoplastic lesions. The adrenal glands of these rats were atrophic.

In group IV (intact rats fed DAB), the livers of 20 out of 36 rats surviving for 189 days (55.5 percent) showed neoplastic lesions: 8 rats had fully developed tumors (H) of the liver; 5 rats had atypical liver-cell areas (ALC); and 7 rats had atypical bile-duct formations (ABD).

Percentages of neoplastic lesions of the liver in these four groups are given in table 1.

Moderate and in some cases advanced cirrhosis of the liver, as well as bile-duct proliferation, were present in the rats not treated with DCA (groups I and IV). In the animals treated with DCA (groups II and III), cirrhotic lesions of the liver and bile-duct proliferation were slight and in some instances absent. Fat metamorphosis was remarkably less in DCA-treated rats than in untreated rats.

Adrenal changes.—Regeneration of adrenal cortical tissue was found quite frequently in the adrenalectomized rats: 15 rats (51.7 percent) in group I, and 11 rats (35.5 percent) in group II. The regenerating adrenal nodules were unilateral and were found most frequently in the area of the upper pole of the kidney except in two rats, in one of which the regenerating adrenal cortical nodule was attached to the right lobe of the liver, and in the other to the spleen. Microscopically these nodules consisted of adrenal cortical cells similar to those of the zona fasciculata, and in some of the rats not given DCA, a narrow zone of lightly stained cells was present in the periphery, like those of the zona glomerulosa. In the DCA-treated animals the cells were smaller and deeply stained, like the adrenal cortical cells of intact rats treated with DCA. A regenerating adrenal gland was found in all rats with neoplastic liver lesions. In rats without adrenal regeneration, neoplastic lesions did not develop in the liver. Table 1 shows the relationship between adrenal cortical regeneration and neoplastic liver lesions.

The adrenal glands of DCA-treated rats (group III) showed microscopically a marked symmetrical reduction in the width of the adrenal cortex (fig. 4). The volume of cells of all cortical layers and especially of the zona glomerulosa was significantly reduced. Lipoid droplets disappeared from the cells of the zona glomerulosa, but they were present in the atrophic cells of the zona fasciculata—reduced in several instances to a minimum, however. These features of the adrenal glands of DCA-treated rats were in sharp contrast to the adrenal glands of rats fed DAB but not treated with DCA. Microscopically, the adrenal cortex of the

TABLE 1.—*Comparison of adrenal and liver changes in rats under various treatments*

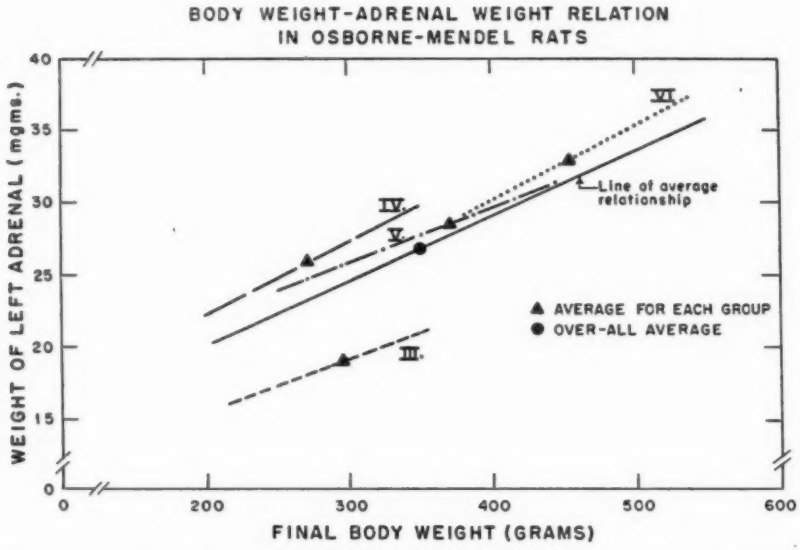
Treatment and food	Rats surviving 189 days	Adrenal condition	Rats with liver lesions	Rats with hepatomas	Group average weight/rat (*gm.*)	Weight of left adrenal (*mg.*)
Group I (adrenalectomy, DAB*)	†29	With adrenal regeneration (15)	12 (80.0%)	2 (13.7%)	302.8 ± 11.05‡	.....
		Without adrenal regeneration (14)	0 (0. %)	0 (0. %)		
Group II (adrenalectomy, DAB, DCA§)	31	With adrenal regeneration (11)	3 (27.3%)	0 (0. %)	314.9 ± 9.64	.....
		Without adrenal regeneration (20)	0 (0. %)	0 (0. %)		
Group III (no adrenalectomy, DAB, DCA)	26	Atrophic adrenal cortex	0 (0. %)	0 (0. %)	295.8 ± 6.33	19.0 ± .426‡
Group IV (no adrenalectomy, DAB)	36	Well developed adrenal cortex	20 (55.5%)	8 (22. %)	270.3 ± 7.19	25.9 ± .717
Controls						
Group V (no adrenalectomy, synthetic diet)	21	Well developed adrenal cortex	0 (0. %)	0 (0. %)	369.6 ± 13.96	28.6 ± .936
Group VI (no adrenalectomy, regular diet)‖	33	Well developed adrenal cortex	0 (0. %)	0 (0. %)	452.3 ± 7.05	32.9 ± .637

*p-Dimethylaminoazobenzene, synthetic diet.
†Kept on 0.9 percent sodium chloride.
‡Standard error.
§Desoxycorticosterone acetate.
‖Derwood pellets.

animals without DCA was well developed with abundant lipoid droplets in the cells of all cortical layers (fig. 5), even more plentiful than in normal rats fed regular laboratory food (Derwood pellets) (fig. 6).

Average adrenal weight and body weight of the six groups of rats is recorded in table 1. The weight of the left adrenal gland was considered in the statistical analysis. This has been done because there appeared to be a relatively constant weight relationship between the right and left adrenal glands.

The statistically fitted lines relating adrenal weight to body weight for each of the experiment groups are shown in text-figure 1.



TEXT-FIGURE 1.—Group III. Rats fed DAB, synthetic diet, and treated with DCA.
Group IV. Rats fed DAB, synthetic diet.
Group V. Rats fed only synthetic diet.
Group VI. Rats fed regular laboratory food (Derwood pellets).

Statistical analysis [5] of these data indicates a close relationship ($P<0.01$) between adrenal weight and body weight. This ratio is significantly smaller in DCA-treated rats (group III, text-fig. 1), and so absolute adrenal weight in this group is significantly smaller than in the other three groups (table 1).

Discussion

This experiment revealed that completely adrenalectomized male rats fed *p*-dimethylaminoazobenzene (DAB) for 189 days do not develop hepatic tumors. Thirty-four adrenalectomized rats without adrenal

[5] For the statistical analysis we are indebted to Mr. Marvin Schneiderman of the Biometrics Section, National Cancer Institute.

cortical regeneration, with or without desoxycorticosterone acetate (DCA) implants, were free of liver tumors after being fed DAB for 189 days. Thus, no hepatomas were induced by DAB in any animal in which adrenals were either absent or atrophic. Twenty-six intact rats fed DAB and treated with DCA for the same period of time had atrophic adrenals and did not develop liver tumors. The adrenal cortex in the DCA-treated rats was atrophic (table 1), the cells were reduced in size and poor in lipoids (fig. 4). Chronic treatment with DCA produces a deficiency of endogeneous corticoids (*21*), and the atrophy of the adrenal cortex (*22–24*) in DCA-treated animals is in agreement with its diminished secretory activity. Accordingly, complete adrenalectomy or depression of adrenal cortical secretion by DCA impaired the development of hepatic tumors in rats kept for 189 days on a DAB-containing diet. Further experiments with animals fed DAB for a longer period of time will clarify the question whether adrenalectomy or treatment with DCA inhibits permanently the development of liver tumors or only delays the formation of hepatic neoplasia.

On the other hand, of 36 rats with intact adrenal glands, 20 developed neoplastic liver lesions, and 8 (22 percent) of these rats had fully developed hepatomas. Of the 15 adrenalectomized rats not given DCA in which regeneration of adrenal cortical tissue occurred, 12 animals had neoplastic liver lesions, and 2 (13 percent) had hepatomas (table 1). Thus, of 51 animals with intact or regenerated adrenal glands not under the effect of DCA, liver hepatomas were induced by DAB feeding in 10 (about 20 percent) of the rats. Adrenalectomized rats with adrenal cortical regeneration and treated with DCA did not develop liver tumors, and only in three animals (27.3 percent) were neoplastic liver lesions found. All these findings indicate the necessity of the presence of intact adrenal glands for the induction of liver tumors in rats fed DAB. Griffin found that hypophysectomy impairs formation of liver tumors in rats fed azo dyes (*25*). However, treatment of these animals with adrenocorticotropin restores the tumor formation in the liver (*26*). Thus it appears that adrenal cortical hormones produced by the adrenal cortex may be necessary for the induction of liver tumors by azo dyes.

Experiments are in progress on the relationship of other carcinogenic azo dyes, and other compounds that induce tumors in the liver, to the role of the adrenal glands in liver carcinogenesis.

Summary

Adrenalectomized rats without adrenal cortical regeneration, kept for 189 days on a synthetic diet containing *p*-dimethylaminoazobenzene and treated or not treated with desoxycorticosterone acetate, did not develop liver tumors. Intact rats treated with DCA had atrophic adrenal glands and were free of hepatic tumors. Nonadrenalectomized rats not given DCA, and adrenalectomized animals with regenerating adrenal cortical tissue, developed neoplastic liver lesions and hepatomas.

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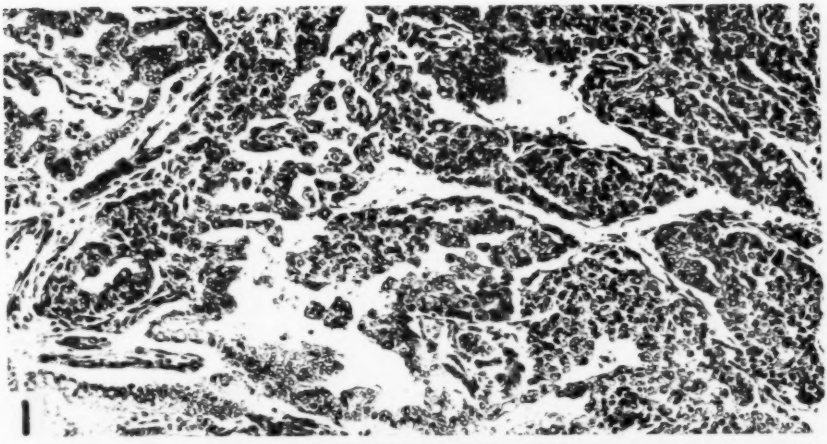
PLATE 57

FIGURE 1.—Liver-cell and bile-duct carcinoma (H). Hematoxylin and eosin. × 145

FIGURE 2.—Area of atypical liver cells and liver cords (ALC). Hematoxylin and eosin. × 260

FIGURE 3.—Area of atypical bile-duct formation (ABD). Hematoxylin and eosin. × 210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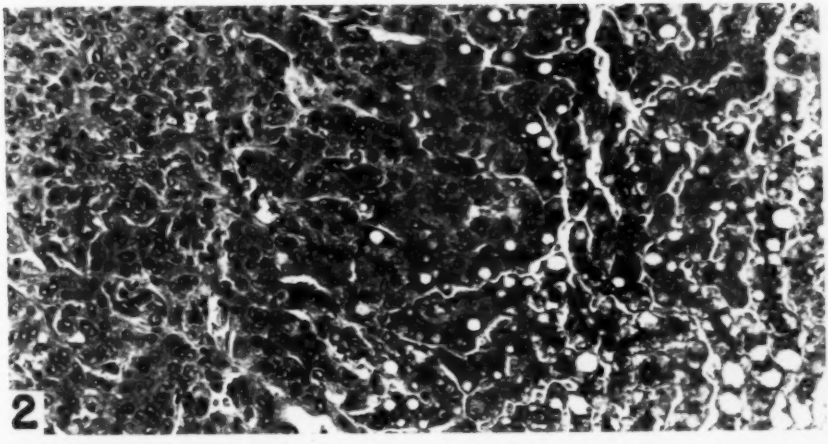
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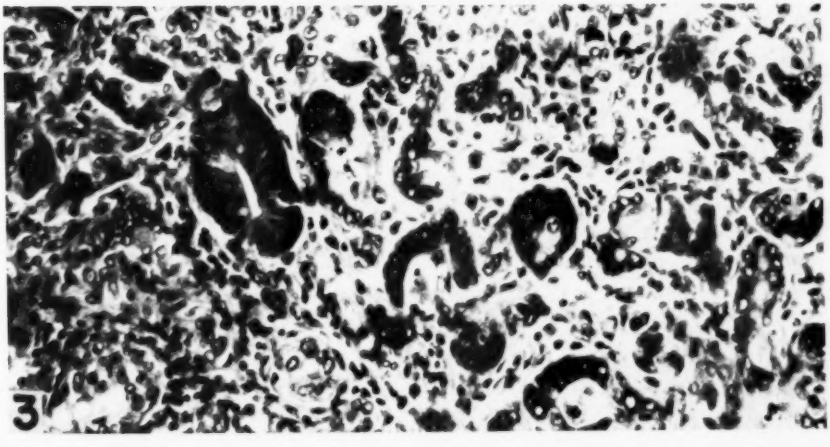
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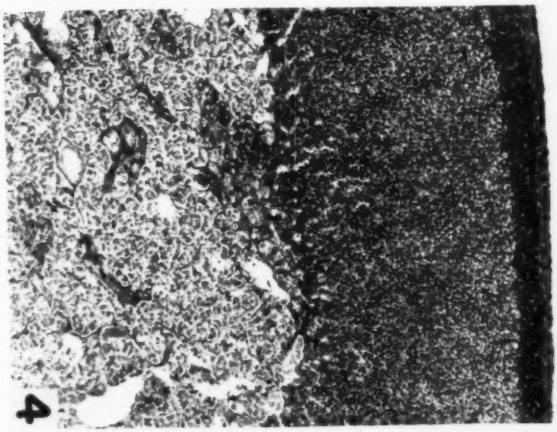
PLATE 58

FIGURE 4.—Adrenal gland of rat fed synthetic diet with DAB and treated with DCA. The width of the adrenal cortex is significantly reduced. All three cortical zones are atrophic, with cells reduced in size and poor in lipoids, especially the deeply stained, compact glomerulosa. Hematoxylin and eosin. × 96

FIGURE 5.—Adrenal gland of rat fed synthetic diet with DAB. Adrenal cortex is well developed with a large zona glomerulosa rich in lipoids in all three cortical zones. Hematoxylin and eosin. × 96

FIGURE 6.—Adrenal gland of rat fed Derwood pellets. Adrenal cortex has the same width as the adrenal in figure 5. Zona glomerulosa and reticularis are less rich in lipoids. Hematoxylin and eosin. × 96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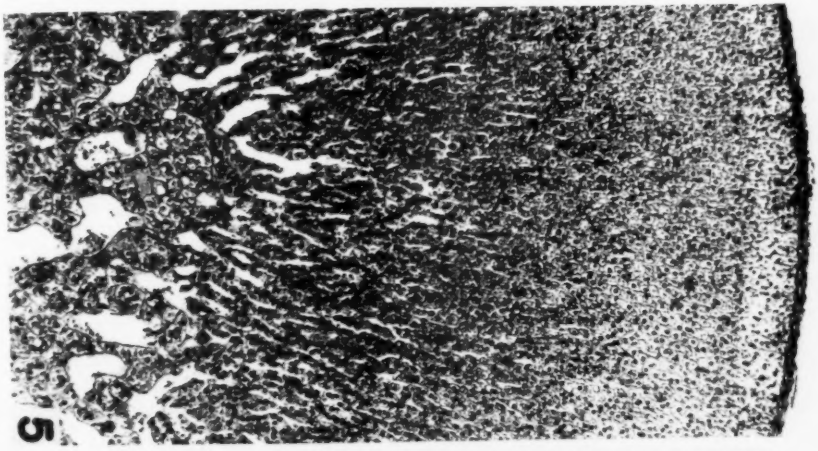
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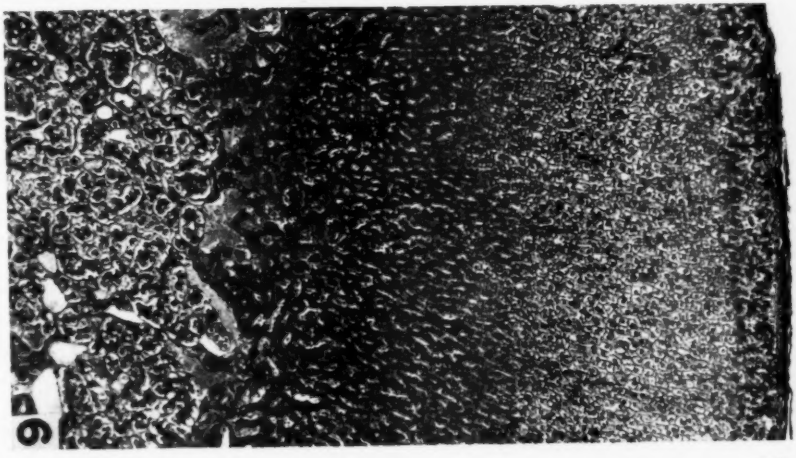
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Fate of Vascularized and Nonvascularized Subcutaneous Homografts in Mice [1]

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Grafts from one individual to another of the same species are usually destroyed by acquired resistance in the host (*1, 2*). Neither the mechanism of this destruction nor the mechanism by which homografts initiate resistance is completely understood. Therefore observations on the changes occurring *in vivo* in homografts are described in the present paper, supplementing both observations reported in histologic studies such as those of Loeb (*3*) and Medawar (*4*) and observations reported in *in vivo* studies (*5*).

Homografts are usually destroyed but destruction may be delayed or prevented under certain conditions (*1, 2*). There are reports in the literature which suggest that nonvascularized grafts survive as homografts (*3, 6–8*). However, certain conditions were present in these experiments that are known or suspected to delay or prevent destruction. That is, the grafts were placed in or bounding the anterior chamber of the eye, or the grafts were of cartilage. Therefore, in the experiments described here, another site and other tissues were used. Under these conditions nonvascularized grafts survived indefinitely, because they did not initiate resistance. Late vascularization, as well as a lack of vascularization, was found to have an effect on the survival of homografts for it delayed and in some cases seemed to prevent destruction.

Materials and Methods

The transparent-chamber technique described by Algire and Legallais (*9*) was used in making most of the observations. Over 100 transparent chambers were installed. Grafts were placed in male mice most of which were from 3 to 5 months old, although a few were as young as 2 months and a few as old as 12 months. Inbred mice of the following strains were used: C3H/HeN, C57BL/6JN, DBA/2N, BALB/cAnN, and A/LN (*10*). In this paper these strains are referred to as C3H, C57BL, DBA, BALB/c, and A respectively. Mice from a strain A colony in which an accidental outcross with BALB/c mice was suspected are referred to as A?. The

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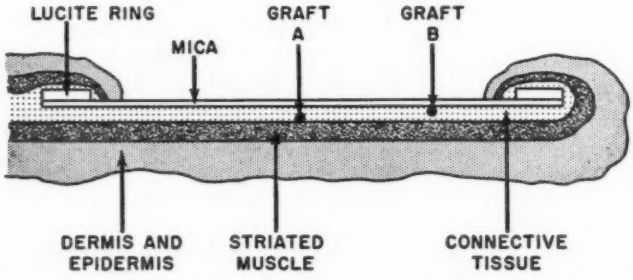
offspring from crosses between BALB/c and A? mice were sometimes used as hosts.

Most of the grafts were of thyroid or harderian-gland tissue. The harderian gland was chosen because of its large branched pigment cells (fig. 1). A few grafts were taken from 15-day-old embryos but most were from newborn mice within 3 days after birth. Older mice were not used as donors because their harderian-gland pigment cells did not survive after transplantation, even in intrastrain grafts. Tissue to be grafted was removed within a few minutes after the donor mouse was killed. The graft was prevented from drying by keeping the humidity of the room near 100 percent. Grafts were small enough to survive without vascularization. The average of the two diameters of the grafts that were in the plane of the surface of the cover-slip of the transparent chamber ranged from 0.4 to 1.6 mm. The thickness of the graft was usually less than this. When more than one graft was placed in a transparent chamber, the grafts were placed at different sites in the chamber area. To obtain grafts that would become vascularized the tissue was placed, by means of glass hooks, close to the subcutaneous muscle layer of the host (graft *A*, text-fig. 1); however, as much connective tissue as possible was left between the muscle and the graft (graft *B*, text-fig. 1) if it was intended that the graft should not become vascularized.

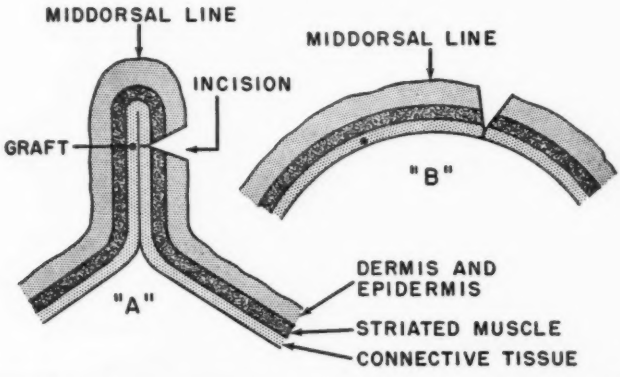


TEXT-FIGURE 1.—Diagram to show the position of grafts placed so that they would become vascularized (graft *A*), compared with the position of those placed so that they would not become vascularized (graft *B*). The positions are shown in relation to the subcutaneous muscle layer and the connective-tissue layer of the host, and in relation to the mica cover-slip of the transparent chamber.

A few grafts were implanted after the transparent chamber was inserted. A small hole was cut in the mica cover-slip of the chamber and the graft was pushed, by means of glass hooks, to a position several millimeters away from the edge of the hole. The hole was completely covered by a mica disc, which became sealed in place when the tissue fluids of the host dried.

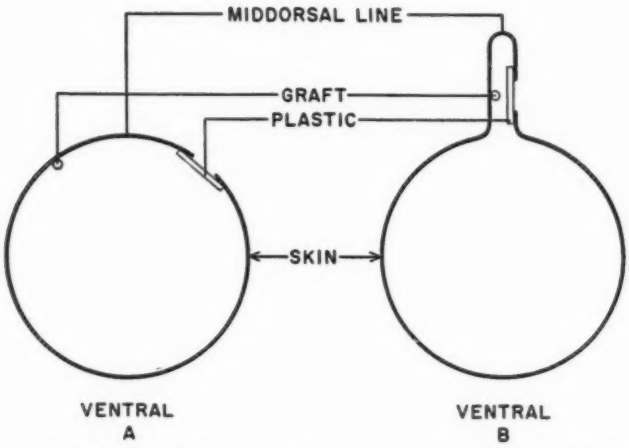
About 20 grafts that were to be nonvascularized were transplanted a considerable time before a transparent chamber was installed or before the graft was used in experiments. When the tissue was transplanted,

the skin of the host was held in place as if a chamber were to be inserted; that is, the skin was pulled up along the middorsal line to form a double fold approximately 15 mm. high. The graft was introduced through an incision in one layer of skin into the subcutaneous connective tissue of the other layer of skin (text-fig. 2, diagram *A*). When the skin was allowed to return to its normal position the graft was on one side of the mid-line and the incision was on the other (text-fig. 2, diagram *B*). However, if the graft was accidentally placed in the connective tissue of the incised layer, then it remained at the incision and became vascularized. Almost half of the first grafts made with this technique stayed at the incision, but after practice nearly all grafts were placed correctly.



TEXT-FIGURE 2.—Diagrammatic cross section of the dorsal skin of a mouse at mid-body, showing how grafts were placed when a transparent chamber was not installed at the same time. The position of the skin and of the graft at the time the implant was made is shown in *A*, and the position after the skin was allowed to return to its normal place is shown in *B*. The graft was placed in the connective tissue belonging to the intact layer of skin.

If only conspicuous changes were to be observed, such as vascularization of grafts or disintegration of pigment cells in grafts of harderian-gland tissue, a transparent chamber was not needed and a simpler method for observing grafts *in vivo* was devised, using a small piece of plastic as a window. The operation was the same as that just described except that after the skin had been allowed to return to its normal position, a circular piece of plastic was inserted through the incision into the subcutaneous connective tissue beneath the muscle (text-fig. 3, *A*). The plastic was centered under the incision. A week after the operation, both graft and plastic were adherent to surrounding connective tissue and the skin could be pulled up until the plastic was opposite the graft, as in *B*. Grafts could be observed through this plastic window at any time. When the skin grew over the opening another incision could be made, so that the window lasted as long as was desired. Results in 25 mice with this technique did not differ from those with the transparent chamber.



TEXT-FIGURE 3.—Diagrammatic cross section of a mouse at the middle of the body to show the relationship of graft and plastic window to the host skin. In *A* the skin is in its normal position with the graft on one side of the mid-line and the window on the other side. At the time the graft was implanted and when the graft was observed through the window the skin was in the position shown in *B*.

Observations and Results

Vascularized Grafts

Almost all the grafts placed close to the host muscle layer became vascularized. Prior to vascularization the blood vessels of the host underlying the graft became enlarged and convoluted, sometimes conspicuously and sometimes to a hardly detectable degree. Vascularization usually took place within the first week after implantation. As long as there was only one connection between the vessels of the graft and of the host, blood entered the graft but could not get out and therefore the graft vessels became engorged with the host's blood (fig. 2). If grafts of harderian gland remained engorged for more than four or five days, the pigment cells disintegrated. The disintegration began in the center of the graft. Since in nonvascularized grafts in resistant hosts disintegration began at the periphery of the graft (as described in the next section), it is probable that the cells in engorged grafts succumbed to a low concentration of oxygen or a lack of nutrients rather than to acquired resistance in the host. Similar changes probably occurred in the harderian-gland acinar cells and in thyroid follicle cells, but the harderian-gland pigment cells were the only ones in which the changes were not obscured by the engorged vessels. Most grafts remained engorged for less than one or two days before other connections between host and graft vessels were established and circulation began.

Intrastrain grafts showed little change after circulation started. In interstrain grafts, within a few days after vascularization leukocytes began to stick to the walls of the vessels. They accumulated in gradually increasing numbers, first in the veins (fig. 3), then in the capillaries, and

later in the arteries. Host cells, presumably leukocytes, accumulated around the grafts. Functional vessels in the grafts gradually became fewer in number (fig. 4). In some cases the leukocytes sticking to the vessel walls seemed to cause occlusion, but whether or not that was the cause of the ensuing stasis was not clear. Stasis often developed throughout a graft, but occasionally only a few areas showed stasis at any one time, or only a few areas showed stasis at first and later the entire graft became involved. It is of interest that in grafts of harderian-gland tissue, pigment cells disintegrated before stasis developed. Also, the pigment cells in well vascularized areas disintegrated sooner than those in poorly vascularized areas. The disintegration of follicles in grafts of thyroid tissue could not be followed as closely as that of the harderian pigment cells because they were more easily obscured by the accumulation of the host's cells. After stasis had occurred throughout a thyroid graft, follicular outlines were sometimes seen although one could not tell whether the cells were still alive.

At the edge of a graft showing stasis the functional vessels looped back sharply, because when circulation stopped in the graft the blood from afferent vessels was diverted to nearby efferent ones through the first connecting vessel present outside of the graft. Within the graft the vessels were either empty of blood or were filled with noncirculating blood (fig. 5). Sometimes there was no circulation in grafts for as long as ten days. When circulation returned the vessels were fine, the network sparse, and corpuscles did not stick to the vessel walls. The graft lost all trace of its original structure and appeared as a granular mass which gradually disappeared.

In comparing the periods of time required for destruction of grafts in different hosts, the interval between vascularization and destruction was taken rather than the interval between transplantation and destruction. This was done because our observations (see following section) showed that nonvascularized grafts did not initiate resistance in the host. If two or more grafts were placed in one host at the same time and one of the grafts was vascularized before the other, the interval before destruction was measured, for both grafts, from the time the first graft was vascularized. The time of vascularization given in the tables is based on the day circulation started in the graft rather than on the day of engorgement. The graft was considered to be destroyed, in the case of harderian-gland tissue, when no expanded pigment cells were left, and in the case of thyroid and other tissues, when stasis was observed throughout the graft.

The period of survival of homografts was not significantly affected by differences in the size of the grafts or in the age of the donors, or, as can be seen in table 1, by differences in the kinds of tissues grafted or in the strains used as donor or host. However, as will be shown, a striking increase in the time interval from vascularization to destruction was observed if the interval from transplantation to vascularization was over two weeks instead of one week or less.

TABLE 1.—*Fate of vascularized homografts*

Strain of donor mouse	Strain of host mouse	Vascularization during the 1st* week after grafting: Number of hosts	Kind of tissue	Interval between vascularization and destruction (*days*)	Vascularized during the 3d to 5th weeks after grafting: Number of hosts	Kind of tissue	Interval between vascularization and destruction (*days*)
C3H	BALB/c	9	Thyroid	9, 9, 10, 10, 10, 12, 13, 13, 14	2	Thyroid	>58, >161
		6	Thyroid and	10, 10, 15, 20, ?, 25	2	Thyroid and	45–54†, 50†
			Harderian gland	9, 10, 13, 20, >22, >21		Harderian gland	45–54†, 38†
		1	Seminal vesicle	12			
C3H	C57BL	3	Thyroid	6, 9, 13			
		1	Thyroid and	9			
			Seminal vesicle	9			
		1	Harderian gland	7			
C3H	A?	1	Thyroid and	14	3	Thyroid and	25–100†, >115†, 58†
			Harderian gland	14, 17		Harderian gland	25–100†, >115†, 24†
					1	Harderian gland	>51
C3H	A	2	Thyroid	12, 15			
		1	Thyroid and	36–95			
			Harderian gland	36–95			
		2	Harderian gland	<8, <8			
C3H	BALB/c ×A?	1	Thyroid	17	1	Thyroid and	27–90
		1	Harderian gland	13		Harderian gland	27–90
C57BL	C3H	3	Thyroid	8, 10, 12	1	Thyroid and	>75†
		9	Harderian gland	10, 11, 13, 13, 14, 16, 25, >22, >24		Harderian gland	25–75†
DBA	C3H	20	Thyroid	8, 8, 8, 10, 11, 11, 11, 11‡, 12, 12, 13, 13‡, 14, 14, 15‡, 17, 17, 17, 17, 25‡	1	Thyroid	>130†
					1	Thyroid and	>40†
						Harderian gland§	>40†

*Since a comparison is made in the text between grafts vascularized in the 1st week and those vascularized in the 3d, 4th, or 5th weeks, the following grafts vascularized during the 2d week are not included in the table. Three hosts received grafts of both thyroid and harderian-gland tissue; the grafts of thyroid tissue were destroyed in 8, 12, and 114 days, and the corresponding grafts of harderian gland were destroyed in 8, 12, and 14 days, respectively. Two hosts received one graft of harderian gland each, which were destroyed in 18 and 28 days.

†Part of the host's thyroid gland was removed after the graft was vascularized or after the graft had been in the host for over 2 weeks.

‡Part of the host's thyroid gland was removed 7 days before the graft was implanted.

§Although branched harderian pigment cells were not seen in DBA harderian glands, one could tell that the graft was surviving because vessels could be seen in the translucent harderian-gland tissue.

Most of the grafts were vascularized during the 1st week after grafting. Only a few were vascularized during the next few weeks. No satisfactory method was worked out that would cause a nonvascularized graft to become vascularized at a given interval after transplantation. Of 78 hosts, 61 had one or more grafts vascularized during the 1st week, 5 had grafts vascularized during the 2d week, and 12 had grafts vascularized during the 3d, 4th, or 5th weeks.

For purposes of contrast, grafts vascularized in the 1st week will be compared with those vascularized during the 3d to 5th weeks. Too few grafts were vascularized during the 2d week to distinguish with certainty when the change affecting survival time occurred. The following comparison omits certain grafts included in table 1, namely grafts of thyroid tissue in hosts from which about ⅞ of the thyroid gland had been removed (for purposes unrelated to this paper).[3] Harderian grafts in partially thyroidectomized hosts are included on the assumption that their survival was not affected. All 12 of the grafts vascularized in the 3d to 5th weeks—excluding thyroid grafts in partially thyroidectomized hosts—survived for a minimum of 24 days, whereas of 66 grafts vascularized within the 1st week only 8 survived that long (table 1). In general, grafts vascularized in the 3d to 5th weeks survived several weeks or more longer than did grafts vascularized in the 1st week.

Because it was difficult to cause grafts to become vascularized at desired intervals after grafting, no further studies were made on the effect of late vascularization on grafts vascularized *in situ*. However the vascularization of grafts after various intervals in a foreign strain could be induced by retransplanting the grafts so they were closer to the host vessels. Studies of grafts vascularized after retransplantation compared with grafts vascularized *in situ* should show whether the delay in destruction after vascularization *in situ* was caused by a permanent change in the immunological properties of the graft cells or whether it was caused by a change in the host or in the relationship between host and graft. Retransplantation did not injure the grafts, for if they were not vascularized they survived indefinitely. Seventeen to 353 days after implantation 11 nonvascularized grafts were retransplanted to a second host of the same foreign strain as the first so that they became vascularized within a week. Two grafts survived indefinitely and 9 were destroyed in 24 days or less (table 2). These experiments, although few in number, indicate that most grafts vascularized after retransplantation do not resemble grafts vascularized *in situ*, for in most cases they initiate resistance without delay. It therefore seems probable that the delay in the initiation of resistance after late vascularization *in situ* is not caused by a permanent change in the graft cells; rather it is a change in the host or in the relationship of graft and host.

[3] Evidence (table 1) indicates that the prolonged survival of thyroid grafts is not dependent on partial thyroidectomy. Thyroid grafts that were vascularized late in hosts with intact thyroid glands showed prolonged survival, and thyroid grafts that were vascularized earlier in partially thyroidectomized hosts did not show prolonged survival. Partial thyroidectomy may have some influence on the length of survival of thyroid grafts, for in 3 cases the harderian graft disintegrated sooner than the thyroid graft.

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		1	Seminal vesicle	12			
C3H	C57BL	3	Thyroid	6, 9, 13			
		1	Thyroid and Seminal vesicle	9 / 9			
		1	Harderian gland	7			
C3H	A?	1	Thyroid and Harderian gland	14 / 14, 17	3	Thyroid and Harderian gland	25–100†, >115†, 58† / 25–100†, >115†, 24†
					1	Harderian gland	>51
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		1	Thyroid and Harderian gland	36–95 / 36–95			
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C3H	BALB/c ×A?	1	Thyroid	17	1	Thyroid and Harderian gland	27–90 / 27–90
		1	Harderian gland	13			
C57BL	C3H	3	Thyroid	8, 10, 12	1	Thyroid and Harderian gland	>75† / 25–75†
		9	Harderian gland	10, 11, 13, 13, 14, 16, 25, >22, >24			
DBA	C3H	20	Thyroid	8, 8, 8, 10, 11, 11, 11‡, 12, 12, 13, 13‡, 14, 14, 15‡, 17, 17, 17, 17, 25‡	1	Thyroid	>130†
					1	Thyroid and Harderian gland§	>40† / >40†

*Since a comparison is made in the text between grafts vascularized in the 1st week and those vascularized in the 3d, 4th, or 5th weeks, the following grafts vascularized during the 2d week are not included in the table. Three hosts received grafts of both thyroid and harderian-gland tissue; the grafts of thyroid tissue were destroyed in 8, 12, and 114 days, and the corresponding grafts of harderian gland were destroyed in 8, 12, and 14 days, respectively. Two hosts received one graft of harderian gland each, which were destroyed in 18 and 28 days.

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Because it was difficult to cause grafts to become vascularized at desired intervals after grafting, no further studies were made on the effect of late vascularization on grafts vascularized *in situ*. However the vascularization of grafts after various intervals in a foreign strain could be induced by retransplanting the grafts so they were closer to the host vessels. Studies of grafts vascularized after retransplantation compared with grafts vascularized *in situ* should show whether the delay in destruction after vascularization *in situ* was caused by a permanent change in the immunological properties of the graft cells or whether it was caused by a change in the host or in the relationship between host and graft. Retransplantation did not injure the grafts, for if they were not vascularized they survived indefinitely. Seventeen to 353 days after implantation 11 nonvascularized grafts were retransplanted to a second host of the same foreign strain as the first so that they became vascularized within a week. Two grafts survived indefinitely and 9 were destroyed in 24 days or less (table 2). These experiments, although few in number, indicate that most grafts vascularized after retransplantation do not resemble grafts vascularized *in situ*, for in most cases they initiate resistance without delay. It therefore seems probable that the delay in the initiation of resistance after late vascularization *in situ* is not caused by a permanent change in the graft cells; rather it is a change in the host or in the relationship of graft and host.

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TABLE 2.—*Fate of nonvascularized grafts following reimplantation and vascularization in a new host of the same foreign strain as the first host*

Time in first host before retransplantation (*days*)	Interval between vascularization* and destruction (*days*)	Kind of tissue	Strain of donor mouse	Strain of host mice
17	6	Harderian gland	C3H	A?
21	21, 22	" and thyroid	"	A?
21	7	Thyroid	"	A?
23	11	Harderian gland	"	BALB/c
43	7	"	"	C57BL
52	15	"	"	A?
90†	>109	"	"	BALB/c × A?
125	13	"	"	BALB/c
147	18	Thyroid	"	A?
270	>83	Harderian gland	"	BALB/c
353‡	24	"	"	BALB/c
270	8	"	"	C57BL

*All grafts were vascularized within the first week except the one that had been in a C57BL host for 270 days. It was vascularized on the 9th day.

†Graft had previously been in 3 other hosts of the same foreign strain; in the second host it was vascularized on the 11th day and removed on the 28th day; in the other hosts it was not vascularized.

‡Same graft as described in the line above, 83 days later, and in the 3d host.

Nonvascularized Grafts

When only nonvascularized homografts were present in a host, they survived indefinitely. Ten grafts of thyroid and 15 of harderian-gland tissue survived as long as they were observed in a transparent chamber, that is 25 to 58 days. The 3 grafts that were observed after the chamber was removed were still surviving at 89 to 181 days after grafting. Twenty pieces of harderian-gland tissue were grafted 1 to 9 months before they were included in a transparent chamber. At the time these grafts were implanted the technique used resulted in only about 50 percent nonvascularized grafts. Eight of the grafts were not found and had probably been vascularized and destroyed. Twelve still survived after 4 to 10 months.

Nonvascularized harderian-gland grafts, whether interstrain or intrastrain, that had been in the host for several months showed no change in size. They were often surrounded by fat and were dark with pigment (fig. 6). In addition to branched pigment cells, there were pigment-filled phagocytes unevenly distributed throughout the graft. There was almost no migration of phagocytes out of the graft. In figure 6 some pigment-filled phagocytes are seen outside of the graft; however, this is not because the cells emigrated, but rather the result of movement of the graft in relation to the surrounding tissue when the transparent chamber was inserted. After retransplantation the graft became paler because some of the pigment-filled phagocytes migrated into the surrounding tissues. The phagocytes moved according to the orientation of the host's connective tissue, which guided them parallel to the mica of the chamber

Page 827 (February 1954 issue): 10 lines above *Discussion*, the sentence reading, "New harderian-gland grafts were placed so they would become vascularized in 5 hosts in which . . ." *should read* "New harderian-gland grafts were placed so they would *not* become vascularized . . ." (In the paper "Fate of Vascularized and Non-vascularized Subcutaneous Homografts in Mice," by Merwin and Hill.)

so that only a few reached the vicinity of the host's blood vessels in the underlying layers of tissue. The possible significance of this behavior of pigment-filled phagocytes will be discussed later.

Experiments were carried out to determine whether nonvascularized homografts survived because they did not initiate resistance or because they did not succumb to resistance. In one set of experiments, 16 mice having either transparent chambers or plastic windows were prepared so that each mouse had both a vascularized and a nonvascularized graft. The nonvascularized grafts were always destroyed at the same time as the vascularized ones (figs. 7, 8). It is interesting that cells in the peripheral portions of large, nonvascularized harderian-gland grafts often disintegrated several days sooner than those in the center. In another series of experiments, new harderian-gland grafts were placed in transparent chambers in which vascularized grafts from the same donor strain had been destroyed less than 22 days earlier. Thirteen out of 14 of the new nonvascularized grafts were destroyed within 6 days and 1 was destroyed in 9 days. Both sets of experiments indicate that nonvascularized grafts succumb to resistance initiated in the host by other grafts that are vascularized. One can conclude that these nonvascularized grafts do not initiate resistance for if they did initiate it they would succumb to it.

Experiments were set up to see whether nonvascularized grafts left in host mice for long periods of time would ever initiate resistance or become capable of surviving in resistant hosts. New harderian-gland grafts were placed so they would become vascularized in 5 hosts in which nonvascularized grafts had been present for from 18 to 301 days. The new grafts survived for 34 to 138 days, when the experiments were terminated. One can conclude that nonvascularized homografts do not initiate resistance even if they are left for a long period of time in a foreign-strain host. One can also conclude that nonvascularized grafts do not change with time in a foreign strain in such a manner that they can survive in a resistant host, for after nonvascularized grafts had survived in a host of a foreign strain for 1 to 9 months, they still disintegrated within 3 weeks after a new graft from the same donor strain became vascularized (table 3).

Discussion

The Initiation of Resistance in the Host by Homografts

Nonvascularized grafts did not initiate resistance under the conditions of the present experiments. This conclusion was drawn from the fact that nonvascularized grafts survived indefinitely unless the host was made resistant by another graft that was vascularized. Two other types of grafts are reported in the literature that do not seem to initiate resistance in the host but that do succumb to resistance. These two types of grafts are corneal grafts transplanted to the cornea (*7*) and possibly grafts of a suspension of epidermal cells (*11*). In both cases, it was suggested that the low dose of foreign cells prevented the initiation of resistance, and, in the case of the corneal grafts, it was also suggested that the limited amount

TABLE 3.—*Fate of nonvascularized grafts that had survived in a foreign strain for 2 to 9 months when new grafts from the same donor strain were introduced and some became vascularized*

Time first grafts had been in foreign strain (*days*)	Tissue		Interval after vascularization* of new graft before destruction of grafts (*days*)		Strain of donor mouse	Strain of host mouse
	First grafts	New grafts	First grafts	New grafts		
60	Harderian gland.	*spleen*†.....	‡11	.....	C3H	BALB/c
§{74 / 120	" ..... / "	*harderian gland*..	{14 / >20}	13	"	{BALB/c × A?
{105 / 105	" ..... / "	*thyroid*.....	{14–16 / 14–16}	11–13	"	BALB/c
{110 / 110	" ..... / "	*thyroid*..... / harderian gland.	13–15 / 13–15	9 / 12	"	"
170	"	*spleen*.....	‡11	.....	"	DBA
210	"	*thyroid*.....	15	12	"	BALB/c
{270	"	{*thyroid*..... / harderian gland.	10	{‖ / 10	"	C57BL

*All grafts were vascularized within 1 week after grafting.

† Italics indicate that the graft was vascularized.

‡ The new graft of spleen was not made into the transparent chamber but was placed subcutaneously by trocar. The day of vascularization was not observed, therefore the number of days after the spleen was grafted is given rather than the number of days after its vascularization.

§ Braces signify that the grafts were in the same host mouse.

‖ Thyroid graft was out of sight at the time it disintegrated.

of vascularization and the late vascularization might also have played a role.

An hypothesis to explain why nonvascularized grafts do not initiate resistance is suggested by the observation reported in this paper that very few pigment-filled phagocytes reached the vicinity of the host's blood vessels from nonvascularized grafts. Graft cells, or host cells that have phagocytized them, may behave in the same manner as the pigment-filled phagocytes. It is possible that the entrance of either graft cells or phagocytized graft cells into the host vessels is a necessary step in the initiation of resistance by homografts.

Late vascularization—that is, vascularization during the third to fifth weeks instead of during the first week after transplantation—has been shown to prolong survival subsequent to vascularization by several weeks or in some cases seemingly indefinitely. The longer survival did not seem to be caused by a decrease in the sensitivity of the graft to resistance in the host, for even after nine months nonvascularized homografts were destroyed if a new graft from the same donor strain was implanted and became vascularized. Neither did the longer survival seem to be caused

by a loss of power to initiate resistance, for grafts retransplanted after several weeks or months often elicited immunity. It is therefore suggested that although grafts seem to retain indefinitely the power to initiate resistance, this power becomes less effective or is ineffective after a graft has been *in situ* for several weeks. The entrance into host vessels of graft cells or host cells that have phagocytized them may, it was suggested above, be necessary for the initiation of resistance. This may not occur under the conditions present around a graft that has been in position for several weeks. Billingham and Medawar (*11*) found that the longer grafts of epithelial cells survived, the less was the chance that a graft would regress spontaneously. Woodruff and Woodruff (*12*) reported that only after a period of several months in the anterior chamber of the eye could thyroid homografts be successfully transplanted to the subcutaneous site.

Greene (*13*) suggested that a delay in the vascularization of homografts of tumors in the anterior chamber of the eye explains the fact that takes are often obtained there when they cannot be obtained with subcutaneous implants. He found that a delay in vascularization of 3 days or more provided time during which the donor stroma died, leaving only tumor parenchyma. The parenchyma, he suggested, might not show the same incompatibility with the incoming host stroma that the original stroma would. There is evidence that under the conditions of the present experiments the stroma survived in nonvascularized grafts for many weeks (unpublished work of Algire and Merwin). A comparison of the observations of Browning (*5*) and those reported here suggest that destruction occurs sooner after vascularization when a graft is placed subcutaneously than when it is placed in the anterior chamber of the eye, even when vascularization occurs earlier in grafts placed in the eye. A longer interval before destruction could explain increased takes of tumors in the anterior chamber, for tumors—unlike harderian-gland grafts—may no longer succumb to resistance in the host once active growth begins (*14*).

Destruction of Homografts by Acquired Resistance in the Host

Nonvascularized homografts succumbed to acquired resistance under the conditions of the experiments described here. This is the opposite of the findings of Loeb (*3*), Maumanee (*7*), and Medawar (*8*) on nonvascularized grafts. Loeb found that subcutaneous homografts of ziphoid cartilage survived even in immune hosts. Cartilage might survive, Rogers suggested (*6*), because it is not vascularized. It has been shown here that nonvascularized thyroid and harderian-gland grafts do not survive; therefore cartilage probably survives because of some special characteristic. Loeb suggested that the large amount of matrix might protect the cells. The possibility should also be considered that the graft cells are replaced by host cells. Medawar found that nonvascularized homografts of skin in the anterior chamber of the eye of immunized hosts were not destroyed. Maumanee reported that corneal grafts that had been transplanted for 6 weeks survived in immune hosts. Corneal grafts may survive for the same reasons that grafts within the anterior

chamber of the eye survive, as pointed out by Medawar, or they may survive because the cells have been replaced by host cells, an explanation favored by Maumanee (7). Medawar suggested that nonvascularized homografts in the anterior chamber of the eye survived because "the immune reaction . . . is mediated by blood plasma or by cells transported in it." If elements of the blood are needed for destruction then it is necessary to explain why these elements pass out of the vessels at the subcutaneous site and not in the anterior chamber of the eye. It is concluded that in each of the 3 cases where the survival of nonvascularized homografts is reported in the literature, either a special tissue or a special site is involved and it is likely that most tissues at most sites would succumb to resistance.

In the course of the destruction of harderian-gland grafts, it was observed that pigment cells in well vascularized portions of the grafts disintegrated before those in poorly vascularized portions, and that the cells at the periphery of nonvascularized grafts disintegrated before those in the center. One possible explanation of these observations is that there may be a low concentration of the elements causing destruction, and that they are used up and need continuous renewal from the blood stream. Another explanation is that penetration from blood vessels may be slow.

It is of interest that harderian-gland pigment cells disintegrated before stasis occurred in the graft vessels. It is possible that stasis was caused by the disintegration of the tissue around the vessels, or that the harderian-gland cells may be more sensitive than the endothelial cells of the graft to resistance in the host. Since thyroid follicles could sometimes be seen after stasis occurred, perhaps thyroid cells are less sensitive than endothelial cells.

Summary and Conclusions

A transparent-chamber technique was used to make observations *in vivo* on subcutaneous homografts. Thyroid and harderian-gland tissues from embryo and newborn mice were placed in adult mice of a different strain. Grafts were small enough so that they survived when not vascularized. Both vascularized and nonvascularized grafts were studied.

Homografts vascularized within a week after transplantation were usually destroyed by 12 days after vascularization.

Nonvascularized homografts survived indefinitely unless a vascularized graft from the same donor strain was, or recently had been, present in the host. If a host with a nonvascularized graft that had been implanted for 1 to 9 months received a second graft that did not become vascularized, both grafts survived indefinitely; however, if the second graft became vascularized, both grafts were destroyed. It was concluded that a nonvascularized graft remains sensitive to resistance in the host but it never initiates resistance. A nonvascularized graft can serve as an indicator of acquired resistance in a host; an indicator that does not initiate resistance and can be followed over a long period of time.

Homografts that were vascularized late—that is, during the third to fifth weeks after transplantation instead of during the first week—survived at least 2 weeks longer than most grafts vascularized early, and some seemed to survive indefinitely. However, no such increase in the period of survival usually occurred when nonvascularized homografts that had been in a host for several weeks or months were vascularized early after retransplantation to a new host. It was concluded that the grafts retained the power to initiate resistance but that when vascularization did not take place until late the power was either less effective or was ineffective.

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PLATE 59

FIGURE 1.—Large branched pigment cells in a graft of harderian-gland tissue 15 days after transplantation from a newborn C3H mouse into an A? host. The graft had become vascularized on the 8th day after transplantation (arrow points to a vessel). The pigment cells were completely disintegrated by the 23rd day after vascularization. × 200

FIGURE 2.—Graft of harderian-gland tissue engorged with blood of the host. This intrastrain graft was from a newborn C3H mouse and was transplanted 4 days before the picture was taken. The branched pigment cells can be seen in the less engorged portion of the graft toward the bottom of the figure. Areas of hemorrhage marked by arrows are dark and granular. × 50

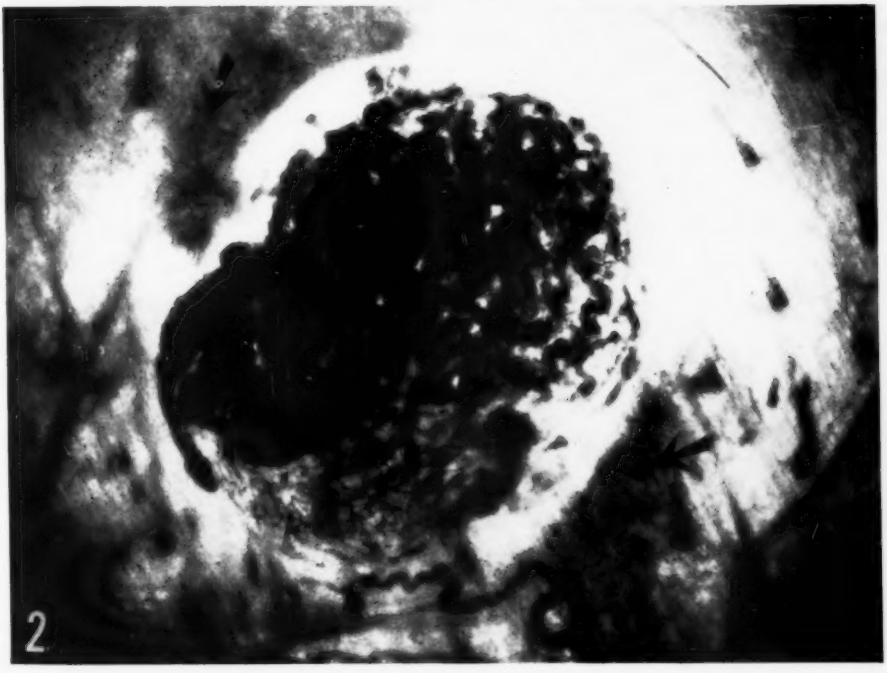

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PLATE 60

FIGURE 3.—Thyroid tissue 45 days after transplantation from a newborn C3H mouse into a BALB/c host. Follicles cannot be distinguished but the vessels outlining them can be seen, as indicated by the arrow in the lower portion of the figure. The graft was vascularized between 12 and 18 days after implantation. Note the large number of leukocytes sticking to the walls of the veins that drain the graft. The upper arrow points to one of the veins. × 150

FIGURE 4.—An interstrain graft of thyroid tissue from a newborn C3H mouse is seen at the top of the figure, and an intrastrain graft of thyroid tissue from a newborn BALB/c mouse is seen at the bottom. The picture was taken 6 days after the grafts were transplanted into a C3H host. The sparser network of vessels in the interstrain graft, compared with the network in the intrastrain one, is typical. × 75

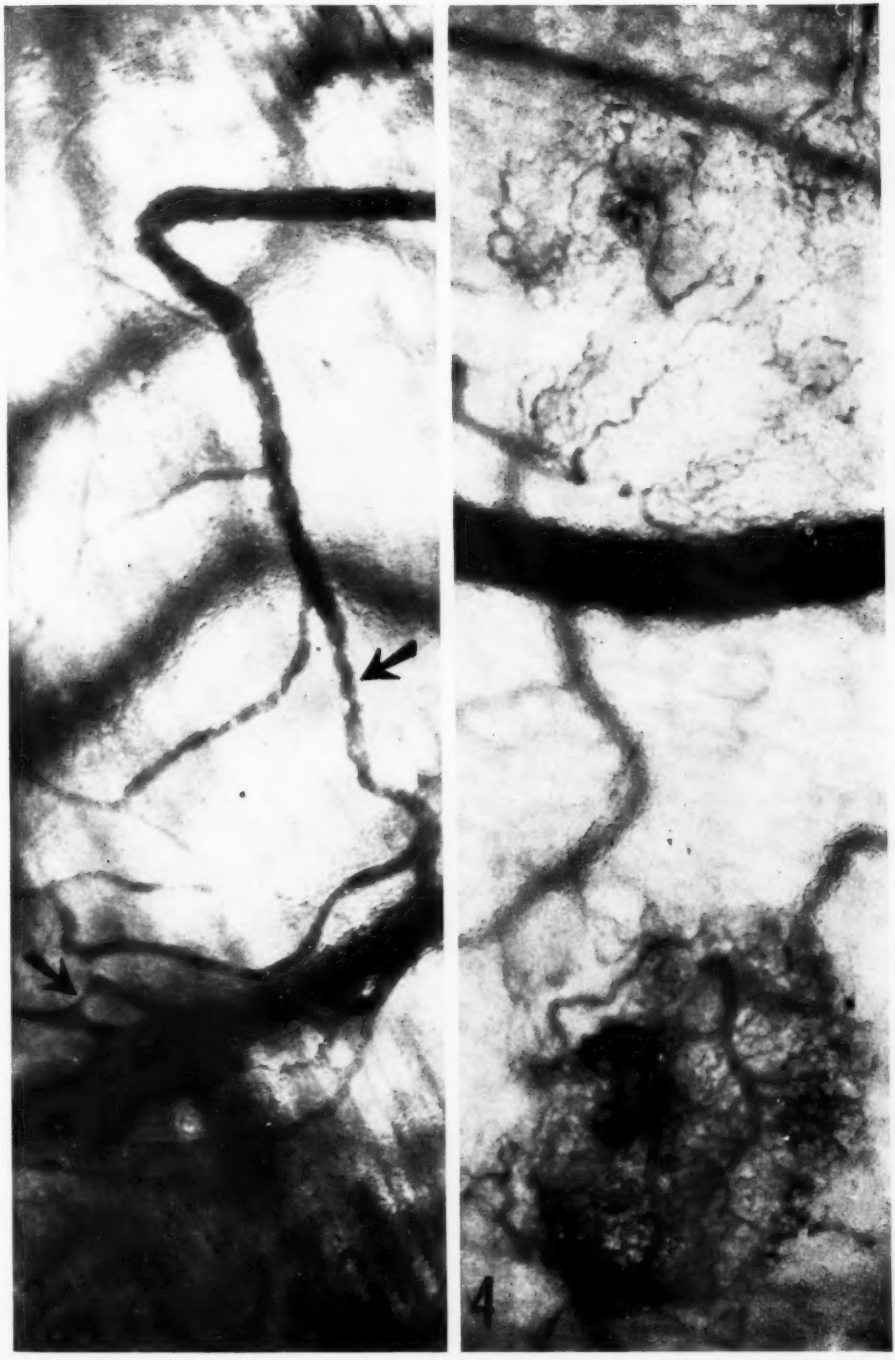
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PLATE 61

FIGURE 5.—Interstrain and intrastrain grafts 12 days after transplantation into a C57BL host. An intrastrain graft of seminal-vesicle tissue from a newborn C57BL mouse is seen at the left of the figure, with vessels encircling it. In the upper right (*arrow*) is an interstrain graft of seminal-vesicle tissue from a newborn C3H mouse. Stasis developed in this graft on the 11th day after transplantation and many red corpuscles were trapped in the vessels. A slightly opaque area can be seen (*arrow at bottom of figure*) at the site of another interstrain graft, a C3H thyroid graft in which circulation started on the 3d day and stasis developed on the 10th day, but in which no blood was trapped in the vessels. × 30

FIGURE 6.—Nonvascularized graft of harderian-gland tissue (from a newborn C3H mouse) that had been in a C57BL host for 120 days. A transparent chamber was installed 11 days before this picture was taken. Clear and refractile fat cells are present on the surface of the graft and make it difficult to distinguish between expanded pigment cells and pigment-filled phagocytes. Note how the arrangement of the pigment cells and phagocytes indicates the acinar structure of the harderian-gland tissue. × 100

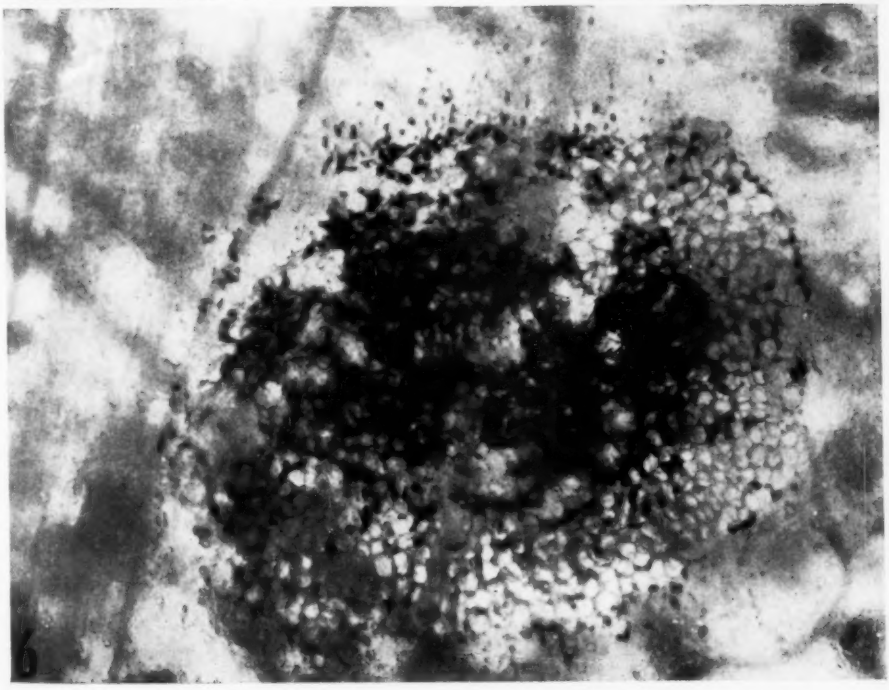
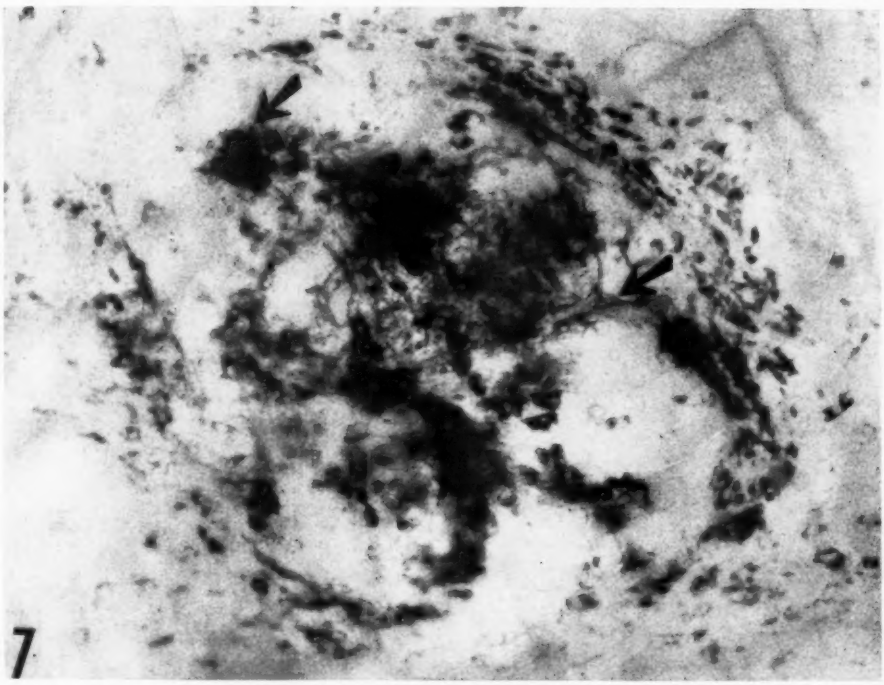


PLATE 62

FIGURE 7.—Nonvascularized graft of harderian-gland tissue from a C3H mouse embryo; the graft had been in one BALB/c host for 30 days and in a second BALB/c host for 19 days. Some of the pigment is in branched pigment cells (*arrow on right*) and some is in phagocytes. These phagocytes sometimes accumulate to form dense patches of pigment (*arrow at top of figure*). Note the manner in which the arrangement of the pigment cells and phagocytes indicates the acinar structure of the harderian gland. × 150

FIGURE 8.—Same graft as shown in figure 7, 13 days later and 10 days after the host received a second graft from the same donor strain—an implant of spleen from a C3H mouse, placed subcutaneously by trocar. Resistance initiated by the splenic graft caused the branched pigment cells to disintegrate. Note that there is no longer any indication of acinar structure in the arrangement of the pigment-filled cells. × 150



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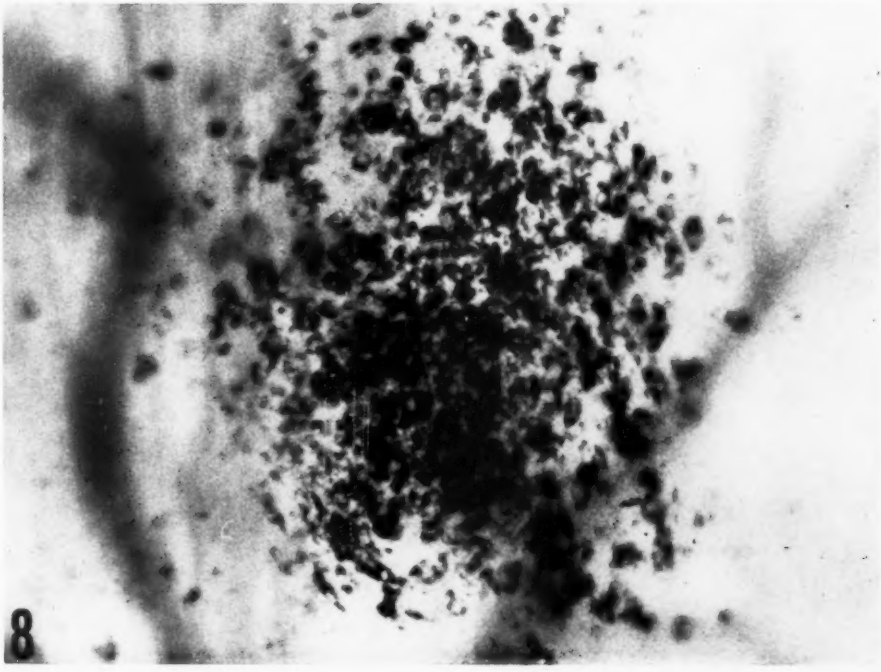

8

Culture Flasks for Use with Plane Surface Substrate Tissue Cultures [1]

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The development and applications of surface substrate[3] methods of tissue culture in this Tissue Culture Section during the last several years (*1–4*) has made necessary the development of more suitable culture vessels. The present paper describes a general type of culture flask that has been fabricated in the Instrumentation Unit of the Laboratory of Biophysics, and which has been useful in work with surface substrate tissue cultures, particularly those used in quantitative studies.

During the period of several years in which these flasks have been in use, a number of changes have been made in their design, due to improved methods of fabrication and of usage. Some of the earlier flask designs were used in experimental work already reported, and were released to other workers for trial. A previously published illustration of one of these flasks [fig. 21 (*5*)] is of older design. In order to avoid any confusion in these various flask designs, the earlier types of the flasks will be briefly described. Since the development of methods for fabrication of these flasks has necessitated certain novel procedures, the methods currently used will be outlined. Certain accessory equipment developed for use with these flasks will also be described.

Requirements of Flask Design

Culture flasks were designed to meet the following general concepts:

1) Two sizes of flasks were necessary. The smaller was needed for experimental work in which a culture area was desired comparable to that obtainable in a Carrel D-5.0 flask. The larger was designed to have a much greater usable culture area and was to be used for already established and rapidly growing stock cultures.

2) The general design of the flasks was such that when flasks were upright, free-floating cells settled out of the fluid, or could be separated

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] The term "surface substrate cultures" will be used to denote cultures in which the cells adhere to and migrate along the surface of a cell support such as a sheet of cellophane or glass, as distinguished from cultures where the cells are embedded in and migrate through a matrix such as a plasma gel.

out by slow centrifugation, while the supernatant fluid could be drawn off and replaced.

3) The optical quality, particularly of the smaller flasks, should allow low-power microscopic examination.

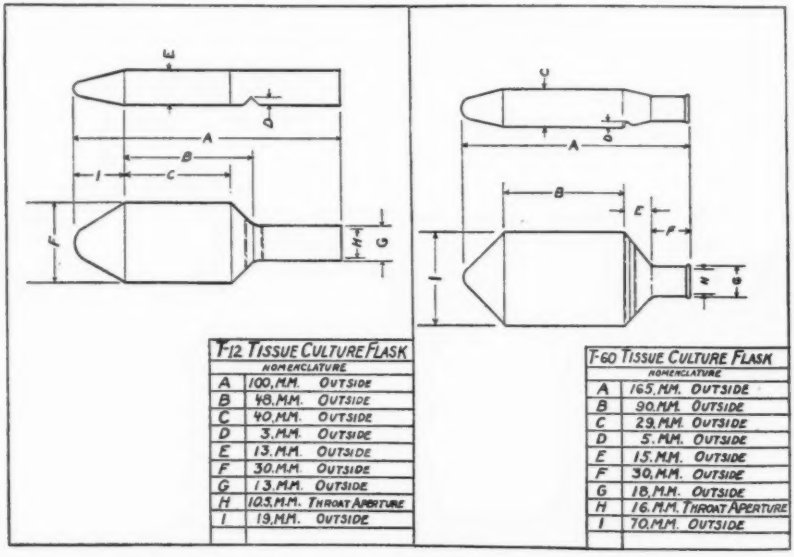
4) Since it was planned to use the flasks in relatively large numbers, it was necessary to keep difficulty of manufacture within reasonable limits.

5) The throat diameter of the flasks was to be sufficiently large to allow instrumentation within the flasks adequate for routine quantitative culture work. For the smaller flasks, a throat diameter uniform with that of the modified Carrel D flasks (*6*) in use was desirable to allow interchangeability of stoppers. For larger flasks a somewhat greater throat diameter seemed necessary.

6) The throat was designed so that the flasks could be readily closed by rubber stoppers of standard size. Throat walls were to be sufficiently heavy to tolerate the routine forcing-in of rubber stoppers to effect a gas-tight seal without breaking the flasks. Length and design of the throat were planned to allow adequate flaming and to keep from wetting the flask stopper unduly with culture media.

In order to identify these various flasks in our records, since they were blown from standard pyrex tubing, they were designated as "T" flasks; the size was indicated by a number showing the approximate floor area of the flask in square centimeters.

The general design and dimensional specifications of the earlier flasks of this general "T" type are shown in text-figure 1. The small and large flasks of this early design were designated as T-12 and T-60 flasks, respec-



TEXT-FIGURE 1.—Early design of T-12 and T-60 flasks.

tively. An even earlier and larger flask designated as the T-72 had the same general shape as this old-type T-60, but was not extensively used.

The two large flat surfaces of the T-12 flask are considered its floor and roof; the floor bears the indented ridge across the throat. This ridge is necessary to keep the culture fluid from running into the flask throat when the flask is laid flat. Thickness of floor and roof was kept as thin as practicable with a mold-blown flask able to withstand light centrifugation, and was about 1 mm. thick for each. This thickness was comparable with that in the Carrel D-5.0 flasks used in this laboratory.

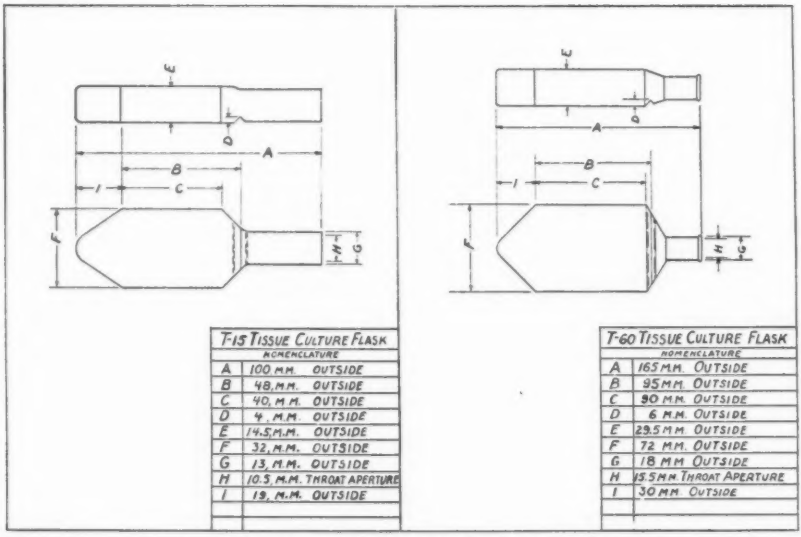
Due to the larger size of the T-60 flask, wall thickness was increased to give mechanical strength. The T-60 flask was designed to be sealed by a #2 rubber stopper. To allow a tight seal the throat was very slightly flared to fit more closely the taper of this size stopper.

Both of these older-type flasks were mold-blown from standard, round, laboratory glass tubing, and their external size and shape were extremely uniform. As the tubing is blown in the mold, the heated tubing stretches unevenly. Therefore, as with all mold-blown bottles, the mid-parts of both the top and bottom of the flasks were relatively thick, while at the corners where the top and bottom joined the sides the glass was much thinner. Flasks fabricated in this way were therefore far from satisfactory since in instances the flask corners were so fragile the flask would shatter on handling or centrifuging. An even more serious defect was that the thickness of the culture-fluid layer over the floor of the flask was very irregular; it was thick along the edges and thin near the middle of the floor. Consequently there was no assurance that living cells adhering to various areas of the surface of the flask floor were under comparable conditions with respect to the media with which they were in contact.

Another criticism that developed in these older flask designs was that the cells adhered to the sloping floor of the tip of the flasks and in some experiments might be above the surface of the culture fluid.

In the later designs this slope of the floor and roof near the tip was therefore eliminated, and the method of fabricating the flasks was radically changed so that instead of being blown in a mold the flask was formed by shrinking on a mandrel. Additional minor changes were made in the flask so that the floor area of the small flask was approximately 15 square centimeters; that of the large flask was 60 square centimeters. These two sizes are designated as T-15 and T-60 flasks respectively, and are shown in text-figure 2.

Just recently two additional sizes of flasks have been found useful. With quantitative studies on rapidly proliferating cultures, it became desirable to have an even smaller flask than the T-15. A number of attempts to obtain a smaller flask by reducing the width showed that flasks narrower than the T-15 had a much greater tendency to uneven distribution of culture fluid over the floor, due to formation of a meniscus which thinned the fluid in the center of the flask and forced the fluid up the sides. The flask design finally adopted, and which is at present giving apparently satisfactory results, uses the same width and thickness



TEXT-FIGURE 2.—Current design of T-15 and T-60 flasks. For additional details see figure 1 and table 1.

as the T-15 flask, and maintains the same angle of the closed end, but reduces the floor area by shortening the body of the flask. This has been designated as the T-9 flask.

The last type so far tried was calculated for a floor area of approximately 30 square centimeters. Since there is less extreme lateral stretching of the glass in fabricating this flask from the round tubing, it is substantially easier to make than is the T-60, and has superior optical qualities. It appears, however, that the T-60 will remain the most useful flask for maintaining cultures of established and rapidly proliferating cell strains. The currently used designs and sizes of these "T" flasks are shown in text-figure 2 and figure 1, and their dimensions are shown in table 1.[4]

Current Method for Fabricating "T" Flasks

In the current method of preparation of these "T" flasks, a blank is prepared for each flask from standard-wall pyrex #7740 tubing. The internal cross-section dimensions and the design of the closed end of this blank are identical with the corresponding internal dimensions of the finished flask. The shoulders and throat of the flask are later formed by hand manipulation of the blank in the flame.

In fabricating the blank for each flask, a piece of tubing of appropriate length and diameter is heated in a broad and bushy flame to a temperature at which it is somewhat plastic. While in the flame it is laterally stretched and flattened by rapidly and consecutively passing down into it, and then

[4] Current designs of these flasks are now commercially available from Robert Kesterson, 5304 Sangamore Road, Washington, D. C., and from Kontes Glass Co., Vineland, N. J.

TABLE 1.—*Dimensions of "T" flasks in mm. with additional details of design*

Flask designation→	T-9	T-15	T-30	T-60
Length of initial tube used for making blank	100	100	165	210
Outside diameter of original tube used for making blank	25	25	35	54
Length of finished blank used for making flask	90	90	135	170
A. Over-all length	80	100	140	165
E. Outside thickness	14. 5	14. 5	20	29. 5
F. Over-all width	32	32	46	72
I. Outside length of bottom angle	19	19	20	30
D. Outside depth of ridge	4	4	6	6
C. Length of straight part of body	25	40	70	90
B. Length from ridge to bottom angle	30	48	80	95
H. Inside diameter of throat, 15 mm. from tip	10. 5	10. 5	12	15. 5
H. Inside diameter of throat, 3 mm. from tip	.....	.....	.....	16. 5
G. Outside diameter of throat, 15 mm. from tip	13	13	15	18
End of throat	straight	straight	rolled bead	heavy rolled bead
Standard rubber stopper size for sealing	00	00	0	2

withdrawing, each of a series of several flat and progressively broader mandrels (text-figure 3 and table 2). The closed end of the blank is formed by flowing the glass over the next to the last mandrel used. In the last stage of formation of the blank, it is shrunk by vacuum onto a polished cast-iron mandrel of exact size to precisely shape the closed end of the flask, and to precisely dimension and highly polish the inner surfaces of the roof, sides, and floor of the flask. While the blank is being shrunk on the mandrel, the heat is raised somewhat and the preparation is hand-worked in the flame to further equalize the thickness of glass over the top, bottom, sides, and pointed end of the mandrel. This final mandrel is then withdrawn and the blank is cooled. In a later operation, after a number of blanks have been prepared, the part of the blank near its open end is reheated in the flame and the shoulders of the flask are then formed on the blank by drawing down and shaping, by blowing and working, the extra length of the blank near its open end. The throat of the flask is then sealed on; the fluid-restricting ridge near the throat is formed by pressure with a V-shaped tool, and the open terminal end of the throat is beaded or fire-polished. The flask is finally oven-annealed by raising the annealing oven temperature to 560° C., shutting off the heat, and gradually allowing the oven to cool to room temperature over a period of 12 hours.

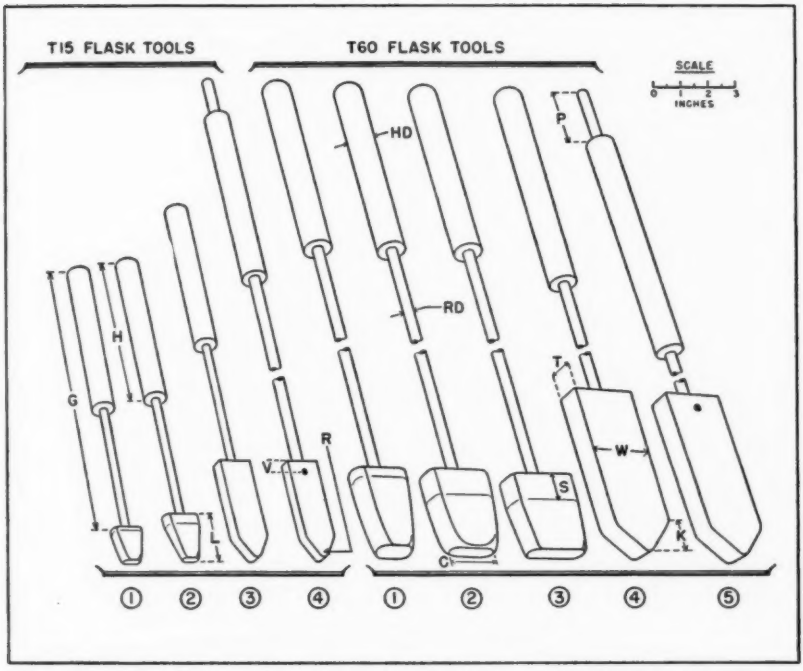
Racks for Incubation and Handling of "T" Flasks

Racks for holding T-15 flasks during incubation are shown in text-figure 4. Racks for other sizes of flasks may easily be worked out for the

TABLE 2.—*Designs of mandrels for shaping flask blanks (all dimensions in inches)*

Designation of flask:	T–9 and T–15				T–30				T–60				
Tool number:	1	2	3	4	1	2	3	4	1	2	3	4	5
Material:	Graphite	Graphite	Graphite	Cast Iron	Graphite	Graphite	Graphite	Cast Iron	Graphite	Graphite	Graphite	Graphite	Cast Iron
Finish:	Ground	Ground	Ground	Ground & polished	Ground	Ground	Ground	Ground & polished	Ground	Ground	Ground	Ground	Ground & polished
Dimensions as indicated in text-figure 3													
L	1⅛	1⅛	3½	3½	2¼	2¼	4¾	5	3	3	3	6¾	6¾
W	1	1 1/16	1. 55	1. 140	1. 4	1. 575	1. 635	1. 615	2 3/16	2 7/16	2⅝	2. 650	2. 630
C	½	½	.....	.....	⅞	1⅛	.....	.....	1¼	1⅜	2⅜	.....	.....
T	. 470	. 475	. 465	. 455	. 670	. 650	. 640	. 625	1. 070	. 955	. 995	. 975	. 960
Radius on corners	3/16	3/16	.....	.....	3/16	3/16	. 010	. 010	½	.....	5/16*	.....	.....
S (straight area length)	⅛	3/16	.....	.....	½	¾	.....	.....	5/16	1	1	.....	.....
G	9½	9½	9½	13	13	13	13	12	15	15	15	12	14
H (handle-wood)	5	5	5	6	6	6	6	6½	6	6	6	7	8
HD	¾	¾	¾	⅞	¾	¾	¾	1	1	1	1	1	1
RD	¼	¼	¼	⅜ OD tube 5/16 ID	5/16	5/16	5/16	⅜	⅜	⅜	⅜	⅜	⅜ OD tube 5/16 ID
K	.....	.....	¾	¾	.....	.....	11/16	.....	.....	.....	.....	1⅛	1⅛
R	.....	.....	15/64	15/64	.....	.....	¼	¼	.....	.....	.....	7/16	3/16
Angle of end	.....	.....	60°	60°	.....	.....	.....	.....	.....	.....	.....	90°	90°
Vacuum hole diameter (both sides of tool)	.....	.....	.....	5/32	.....	.....	.....	.....	.....	.....	.....	.....	¼
P	.....	.....	.....	1¼	.....	.....	.....	1¼	.....	.....	.....	.....	2
V	.....	.....	.....	⅜	.....	.....	.....	3/16	.....	.....	.....	.....	11/16

*Radius on corners for the width of this flask is 1/16. Graphite specification: C. S. or A. T. J., National Carbon Co. Cast iron specification: Fine Grain, Motors and Metals, Inc., 220 West 42d St., New York, N. Y.



TEXT-FIGURE 3.—Design of mandrels or tools used to form flask blanks. The group of 4 mandrels or tools used to form T-15 flask blanks are also used to form blanks for the T-9 flask. The group of mandrels that form the T-30 flask blanks are similar in general shape to those used to form blanks for the T-15 flask. Further specifications of each mandrel or tool are given in table 2. For simplicity, the letter indicating any dimension is shown in the figure for only one tool, but applies to the same dimension for each tool in the figure.

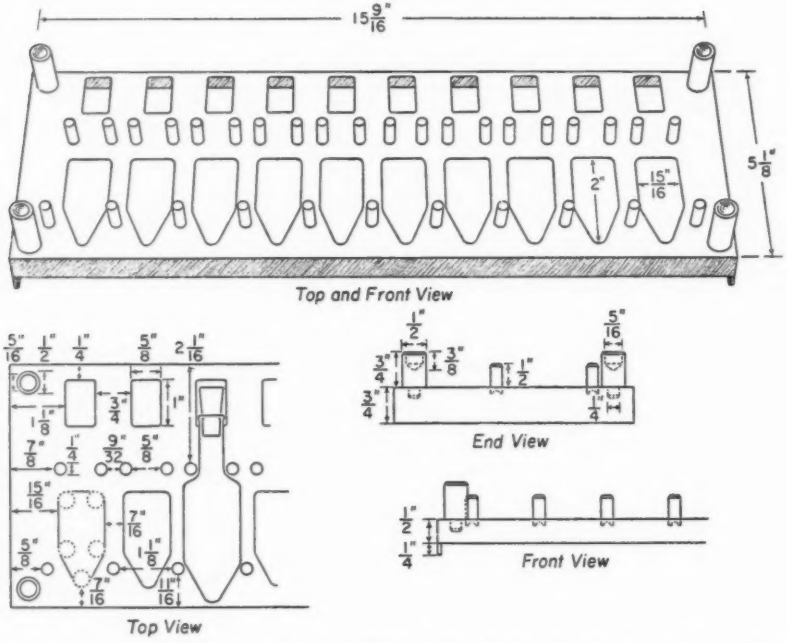
flask size used and the number of flasks desired on each rack. In the incubator, the racks must be laterally separated from each other by several inches to allow air circulation and even temperature control.

A rack for holding T-15 flasks during detailed microscopic examination is shown in text-figure 5. This rack is designed to fit a standard mechanical stage. In use, the pillar of the microscope is slightly tilted.

Discussion

Since the walls of the "T" flasks must stand slow-speed centrifugation,[5] the walls of even the T-9 and T-15 flasks are of necessity thicker than those of the modified Carrel D-3.5 flasks we now routinely use (*6*). Consequently, even these smaller "T" flasks are not designed primarily to attain that perfection of visual and photographic recording of cell morphology which can routinely be obtained with correctly made Carrel D-3.5 flasks. With the current method of fabrication of these smaller

[5] 300 r.p.m. is now routinely used for culture-fluid change and 1,100 r.p.m. for nuclear enumeration.

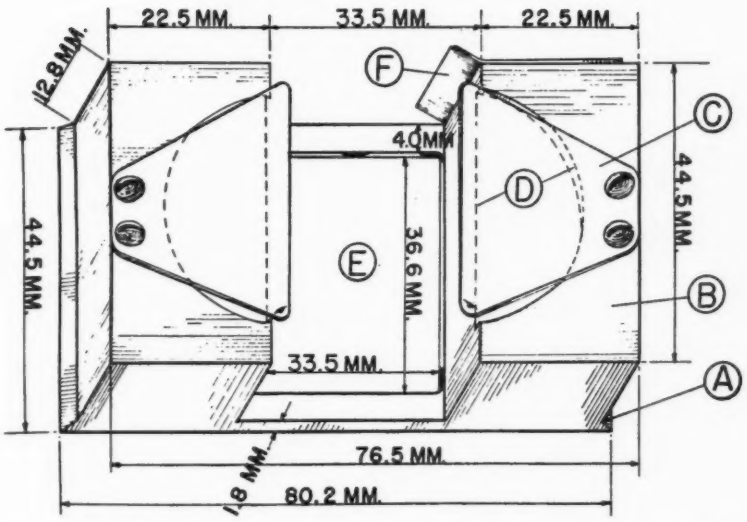


TEXT-FIGURE 4.—Racks for holding T-15 flasks during incubation. Dimensions shown are in inches. These racks are made from 1/16-inch half-hard sheet aluminum. The edges of the rack are turned in a precision brake. The bottom edges of the turned ends of the rack are finally hand-filed, if necessary, so that when the rack rests on them the top surface of the rack is accurately level. The large holes are cut to allow inspection of the flask contents without removal from the rack. The small rectangular holes keep the enlarged free end of the rubber stopper sealing each flask from resting on the rack and tilting the flask out of level. This hole may be omitted in racks for T-30 and T-60 flasks, since the stopper clears the top surface of the rack. The over-all length of the rack accommodates a convenient number of flasks and fits into the incubator used with the long axis of the rack positioned from front to rear in the incubator.

"T" flasks, fair visual or photographic examination of the culture up to about 200 × is practical.

In spite of this optical limitation the T-60 flask has gradually become the standard flask in which stock strains of cells are maintained in this laboratory. By their routine use it is now practical to carry far larger areas of cell sheets and far larger amounts of living cells than was formerly possible; the labor and expense of maintenance of the cell strains are much less than if smaller culture flasks were used.

These "T" type culture flasks, particularly the T-30 and T-60, are of necessity more expensive than, for instance, the usual roller tube. This item of cost will probably make it more desirable for many workers to maintain stock cultures in roller-tube culture vessels in those instances where satisfactory methods for growing the cells directly on the glass or



TEXT-FIGURE 5.—Rack for holding T-15 flask during microscopic examination Dimensions are in millimeters. The body of this rack (*B*) is made from aluminum or dural, while the springs holding the flask in position (*C*) are phosphor-bronze sheet, about ¼ mm. thick. The body is cut out (*D*) to a slight depth under the spring leaf so as not to interfere with it. Ledge (*A*) is to fit the mechanical stage of the microscope; metal piece (*F*) is a limit stop against which the bottom of the flask rests.

under a perforated membrane in such culture tubes can be worked out. However, with some cell types, or with cell population levels that appear to do better with a relatively static culture fluid, the use of the larger "T" type flasks offers definite advantages for the growth of large cultures.

The most significant value of the T-9 and T-15 flasks lies in their usefulness for growth of surface substrate cultures to be used for nuclear enumeration or other comparable quantitative culture methods (*4*, *7*). Their use now makes it practical to carry on accurate quantitative studies of cell proliferation in replicate surface substrate cultures over periods of time which necessitate numerous renewals of the nutrient fluid on the culture.

Summary and Conclusions

A new general type of flask particularly adapted for tissue cultures planted from cell suspensions and growing on surface substrates is described. Four sizes with floor areas of approximately 9, 15, 30, and 60 square centimeters respectively are described, and are designated as T-9, T-15, T-30, and T-60 flasks, respectively. The two smaller sizes are now being used in quantitative studies, while the two larger flasks are routinely used for rapidly growing stock strains of cells or for other work where large quantities of living cells in culture are desired. Characteristics of this flask-type culture, which make it particularly applicable

to quantitative studies involving numbers of replicate cultures and renewals of the culture fluid, are described and discussed.

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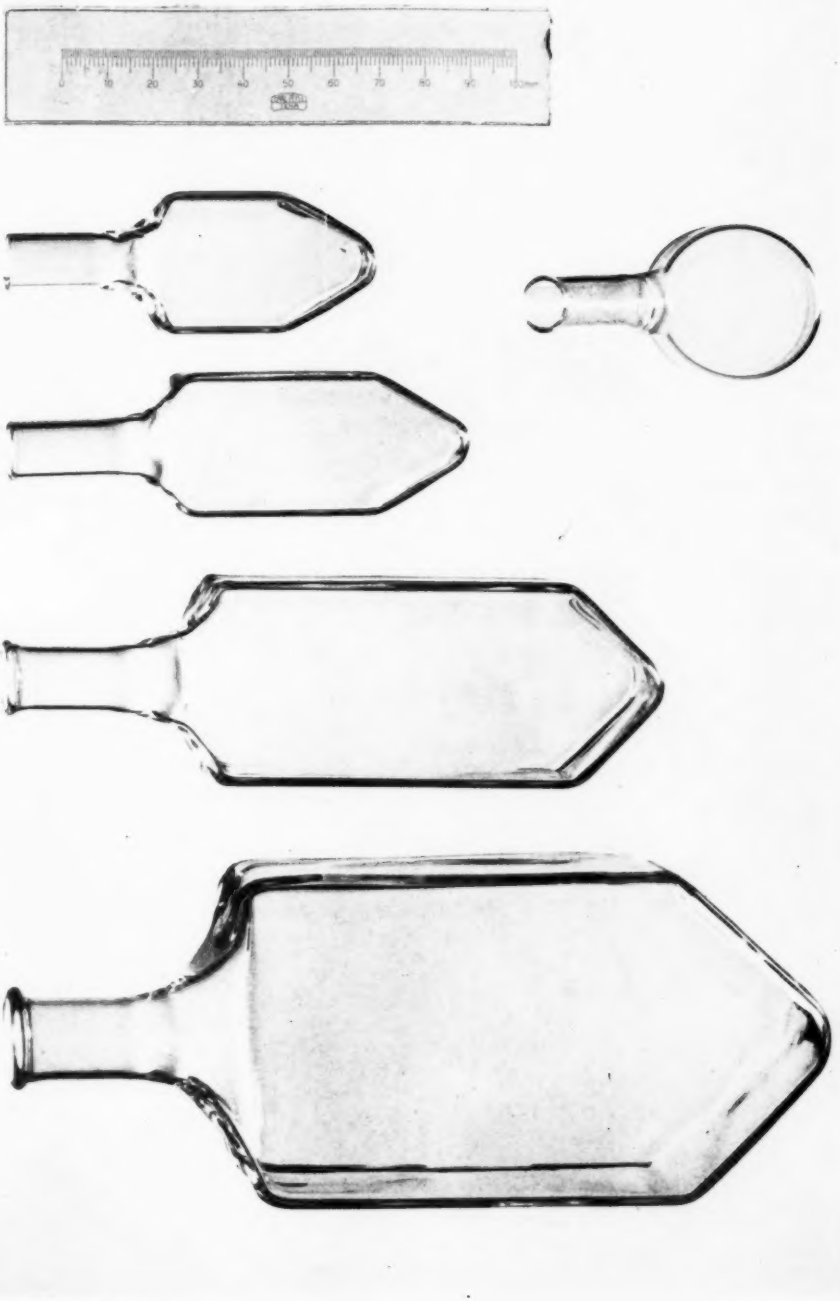
PLATE 63

FIGURE 1.—Photograph showing the 4 sizes of "T" flask currently in use. A modified Carrel D-3.5 flask is shown for comparison.



277342—54——13

Influence of Tube Rotation Velocity on Proliferation of Strain L Cells in Surface Substrate Roller-Tube Cultures [1, 2]

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The rotating or roller-tube type of culture, introduced by Gey and Gey (*1, 2*) for plasma matrix tissue cultures, has come into use in many laboratories. As widely used for plasma-matrix cultures, the tissue explants are embedded in a thin layer of plasma clot on the inner surface of a test-tube-like culture vessel. After the plasma has clotted, a small volume of fluid nutrient medium is added to the tube as a fluid supernatant, the tube is stoppered, and is incubated in an approximately horizontal position in a drum-shaped frame. This frame is kept slowly rotating in a vertical plane around its horizontal axis. As the drum rotates, the tube held within the drum is also rotated, so that the supernatant culture fluid washes over the whole surface of the plasma clot containing the cultures. In this way a simple circulation of the fluid over the cell clumps and the surface of the plasma clot is maintained.

If the rate of rotation of the roller-tube drum is too rapid, the cell colonies tend to become irregularly elongated around the circumference of the tube. With plasma-matrix cultures the increase in the area of the culture is often used as an index of cell proliferation of the culture. Since irregular or elongated cultures are more difficult and less accurate for areal determinations, the tendency has been to use a drum-rotation speed such that the cultures were relatively circular in shape. Thus the usual rotation speed of roller-tube units has been from about 6 to 12 revolutions per hour.

In the present paper data are presented to show that the rate of proliferation of strain L mouse cells planted as a cell suspension and grown on the glass surface of the roller tubes is greatly increased by accelerating the rate of rotation of the culture tube.

Materials and Methods

Roller tubes used were of the design shown in text-figure 1, and were fabricated from pyrex glass. The neck of each tube was constructed so

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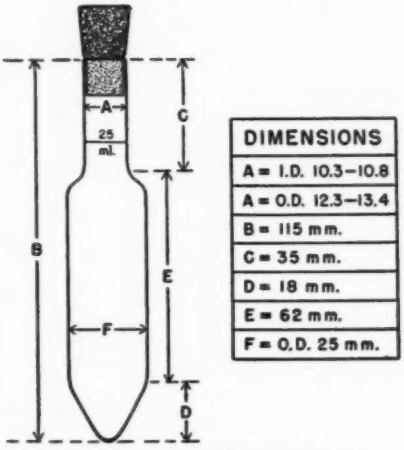
that it could be securely sealed by a size 00 standard rubber stopper. The wall of the tube was 2 mm. thick; the closed end of the tube was cone-shaped so that at each fluid change any loose cells floating in the medium could be thrown out by centrifugation at 300 r.p.m. for 20 minutes, and collected in the tip of the cone. The fluid culture medium was changed periodically, according to the methods previously detailed for quantitative studies on surface substrate replicate cultures (*3*, *4*). The 25-ml. graduation on the neck allowed the same culture flask to be used in fixation and dilution of the nuclei suspension for nuclei enumeration (*4*) if so desired; in the present study, however, the cell suspension was made up to volume in graduated cylinders as originally described (*4*).



TEXT-FIGURE 1.—Design of roller tube.

Roller-tube drum equipment consisted of 8 aluminum-sheet roller-tube drums, each capable of carrying 26 roller tubes of the design shown in text-figure 1. When inserted within the drum each culture tube was clamped by a small spring to keep the tube from rotating on its own axis. These drums were mounted on one frame. The whole assembly was accurately leveled in the incubator so that all tubes were horizontal. This roller-tube equipment was driven by means of a synchronously wound, 1/50th-horsepower, reduction-gear motor mounted outside the incubator so that the heat from the motor did not disturb the temperature control. The drive was by nonslip belt and pulley to the highest-speed roller-tube drum;[4] from this a further reduction nonslip pulley drive operated the next slower drum. In this manner the highest-speed drum was operated at a constant speed of 1,200 revolutions per hour, with the slower drums operating in series from it at 600, 300, 150, 75, 37.5, 18.75, and 9.37 revolutions per hour, respectively. The slowest-speed drum was therefore operating within the usual velocity range of the conventional roller-tube unit.

[4] This was later changed to a sprocket and chain drive.

Temperature control during incubation was maintained by housing the whole roller-tube drum unit in an incubator fitted with a dual system of mercury-in-glass thermoregulators. Air within the incubator was kept in a rapid state of circulation by means of a centrifugal-type fan, running continuously. This fan motor was mounted outside the incubator, and was on vibration absorption cushions so as to rule out cell changes from vibration. Temperature was set at 37.5° C. Before the experiments, regional temperature changes within the incubator were simultaneously checked in 16 representative areas with recording clinical thermometers, and found to be within a range of ±¼° C. Temperature was recorded for the duration of the whole study by means of a chart-recording thermograph capable of recording ±¼° C., and was within the range ±¼° C. This same thermograph would also have recorded any failure in electric current that might have caused the roller-tube mechanism to stop.

Cells used were the pure strain L mouse cells, originated from a single cell isolated in culture (*5*).

Culture medium was a mixture of 40 percent filtered horse serum, 40 percent Earle's saline, and 20 percent 1:1.25 chick-embryo extract from 9-day chick embryos. The embryo extract was prepared by treatment with hyaluronidase, ultracentrifugation, and filtration (*7*). All embryo extract used was less than 10 days old. This was the same culture mixture on which the strain L cells were being routinely maintained. The volume of nutrient medium in each culture was 3 ml. Thrice weekly, the cultures were centrifuged at 300 r.p.m. for 20 minutes. By means of the quantitative methods and equipment previously described (*3*), 2 ml. of the fluid were withdrawn from each flask and replaced by fresh medium. At each fluid change each flask was gassed with 5 percent CO_2 in air and immediately sealed by means of a rubber stopper.

Preparation of replicate cultures was as previously described (*3*). The suspending fluid was the same as the nutrient fluid.

Number of experiments.—A total of three experiments was carried out. The first two experiments tested the action of six different drum speeds on the cultures; the third experiment tested eight speeds. Ten cultures were used for each speed in each experiment. In the first two experiments, 10 control cultures were laid horizontally in a rack in the incubator and neither rotated nor shaken. This made a total of 220 tube cultures in the study. In two experiments, parallel groups of T-15 flask (*6*) cultures, laid flat and not rotated or shaken, were carried as a further control. Since the behavior of the T-15 cultures was similar to that of others in other studies, they were omitted from the third experiment. In each experiment, after 7, 14, and 21 days of growth, at least 3 cultures were randomly selected from each experimental and control group, and were fixed for enumeration of nuclei.

Fixation of cultures and enumeration of nuclei were carried out substantially as previously described (*4*). A few minor facilitating changes were made, however.

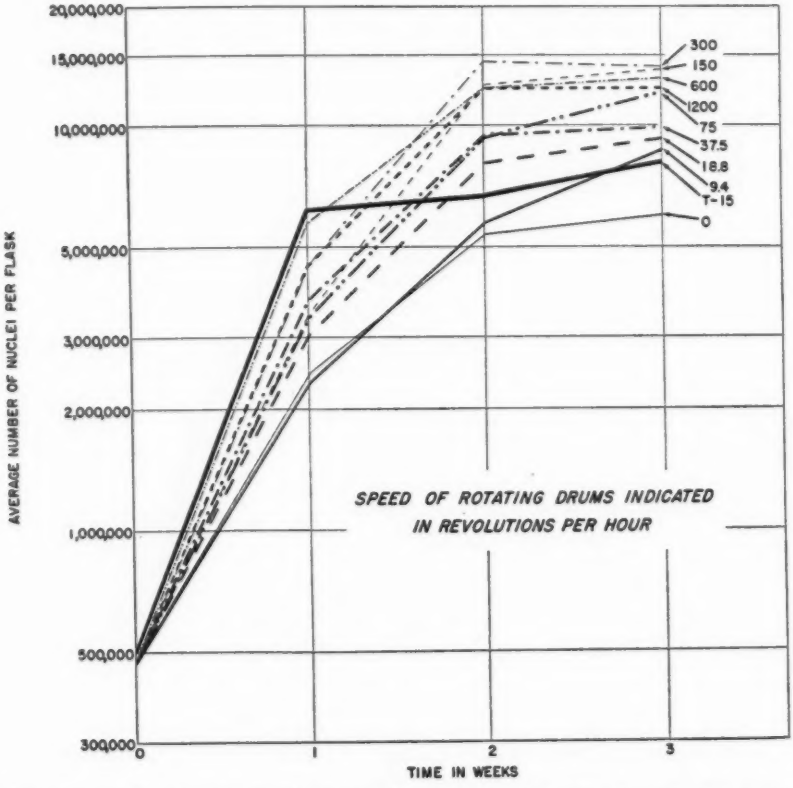
As has previously been shown (*4*), the nuclei-enumeration method used

in these studies records only the intact, normal-appearing nuclei with a normal staining reaction to crystal violet. Nuclei that have undergone such degenerative changes as to be disintegrated, or that do not show the crystal violet staining, are not enumerated. The nuclear counts therefore are to be considered as indicating the number of normal living nuclei in the cultures.

Results

Results obtained are given in table 1 and in text-figure 2. Table 1 gives the number of thousands of nuclei in each culture flask, with averages for each experiment at each drum velocity, and at 7, 14, and 21 days, respectively. In text-figure 2, the numbers of nuclei per culture from all experiments have been averaged for each drum velocity and each time period. The resultant averages have been plotted on a semilog scale against the age of the cultures in weeks.

Specific statistical evaluation was made to compare the influence of culture-vessel design (T-15 flasks versus roller tubes), effect of speed of

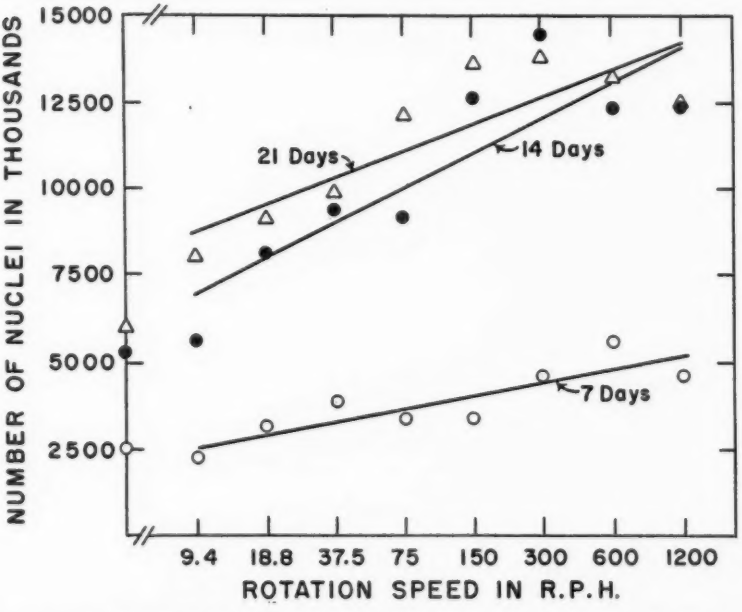


TEXT-FIGURE 2.—Average numbers of nuclei per culture tube or culture flask for each speed of tube rotation and for each time period.

rotation (0 rotation speed versus all other rotation speeds), and to determine possible optimal rotation speed (maximum point of the curve relating total growth to speed).

In the first week, T-15 flasks gave significantly greater proliferation than the nonrotating roller tube (text-fig. 2). At this time the cultures in T-15 flasks appeared to have attained maximum growth, and little additional proliferation appeared to take place. At all times during the experiments, the nonrotating tubes gave the lowest, or next to the lowest, proliferation. The differences in proliferation between nonrotating roller tubes and rotating roller tubes, considered as a group, were statistically significant ($P<.01$) for all three weeks.

For each of the three weeks, straight-line regressions relating total proliferation to the speed of rotation (text-fig. 3) were highly significant (*i.e.* the slope of the line was significantly positive, with $P<.01$). In each of the three weeks, the proliferation at 1,200 r.p.h. was less than was estimated from the fitted straight lines; this leads to the strong suspicion that there is a leveling off, or a down-turn, at highest rotation speed. The apparent down-turn was greatest at three weeks, and least at one week. Curvilinear regressions fitted to these data did not significantly improve the straight-line fits, so that it is not possible to make a firm statement about optimal rotation speeds. However, it appears that maximum proliferation occurred somewhere within the range 300 to 1,200 r.p.h.



TEXT-FIGURE 3.—Straight-line regressions relating total proliferation to the speed of rotation for each of the three weekly periods.

TABLE 1.—*Number of nuclei (in thousands) in each flask, with averages for each experiment at each drum velocity and at each time*

Drum speed (*r.p.h.*)	Experiment No.								
	1410			1426			1506		
	Time after planting in days								
	7	14	21	7	14	21	7	14	21
	Numbers of nuclei in each flask, in thousands								
0	2, 694	4, 204	5, 703	1, 793	5, 587	6, 567	.....	.....	.....
	2, 692	4, 027	6, 063	2, 619	4, 520	5, 706	.....	.....	.....
	2, 926	4, 698	5, 871	.....	6, 182	6, 096	.....	.....	.....
Average	2, 771	4, 310	5, 879	2, 206	5, 430	6, 123	.....	.....	.....
9.4	3, 427	4, 204	9, 096	1, 704	6, 969	6, 427	1, 964	5, 360	8, 408
	2, 342	6, 138	8, 760	2, 093	8, 328	7, 167	2, 322	6, 742	11, 064
	2, 269	5, 213	9, 712	2, 551	4, 409	7, 647	2, 259	4, 453	10, 136
Average	2, 679	5, 185	9, 189	2, 116	6, 569	7, 080	2, 182	5, 518	9, 869
18.8	3, 911	12, 000	7, 866	1, 702	7, 020	7, 580	3, 496	9, 112	11, 936
	2, 040	3, 356	8, 718	2, 353	5, 377	8, 387	3, 633	13, 008	11, 160
	3, 757	7, 888	.....	2, 474	4, 858	8, 647	3, 922	9, 984	10, 952
Average	3, 236	7, 748	8, 292	2, 176	5, 752	8, 205	3, 684	10, 701	11, 349
37.5	.....	.....	.....	3, 594	8, 448	12, 000	3, 619	10, 584	6, 878
	.....	.....	.....	3, 834	7, 605	8, 896	3, 607	11, 408	12, 008
	.....	.....	.....	4, 000	7, 208	10, 016	3, 603	11, 336	.....
Average	.....	.....	.....	3, 809	7, 754	10, 304	3, 610	11, 109	9, 443

75	1, 156	3, 916	11, 528	5, 640	5, 387	9, 904	2, 996	10, 088	13, 600
	3, 018	9, 408	10, 288	4, 289	8, 656	9, 760	3, 973	13, 648	13, 560
	1, 398	7, 296	12, 736	3, 804	11, 264	11, 552	3, 948	14, 568	15, 464
Average	1, 857	6, 873	11, 517	4, 578	8, 436	10, 405	3, 639	12, 768	14, 208
150	1, 971	9, 336	12, 120	.....	.....	.....	4, 844	14, 960	15, 728
	1, 161	10, 128	12, 056	.....	.....	.....	4, 881	15, 568	15, 200
	3, 533	10, 512	11, 888	.....	.....	.....	4, 089	15, 120	.....
Average	2, 222	9, 992	12, 021	.....	.....	.....	4, 605	15, 126	15, 464
300	.....	.....	.....	5, 831	11, 184	12, 160	3, 454	17, 600	14, 720
	.....	.....	.....	5, 280	13, 240	12, 408	2, 314	16, 200	18, 272
	.....	.....	.....	4, 636	12, 816	13, 008	5, 548	15, 712	13, 168
Average	.....	.....	.....	5, 249	12, 413	12, 525	3, 772	16, 504	15, 387
600	5, 555	12, 040	12, 160	.....	.....	.....	6, 139	12, 592	15, 224
	5, 589	13, 200	12, 064	.....	.....	.....	6, 021	10, 376	10, 896
	6, 070	12, 016	12, 648	.....	.....	.....	5, 248	14, 312	16, 808
Average	5, 738	12, 419	12, 291	.....	.....	.....	5, 803	12, 427	14, 309
1200	5, 170	11, 936	14, 304	2, 859	12, 288	10, 736	4, 880	14, 400	15, 544
	4, 150	10, 336	13, 544	4, 898	12, 576	7, 634	5, 181	13, 960	14, 672
	4, 070	10, 592	12, 520	4, 440	11, 480	.....	5, 133	14, 304	13, 856
Average	4, 463	10, 955	13, 456	4, 066	12, 115	9, 185	5, 065	14, 221	14, 691
Static T-15 flask	5, 781	7, 296	8, 648	6, 587	6, 311	6, 023	.....	.....	.....
	6, 606	6, 984	8, 912	5, 636	4, 849	7, 545	.....	.....	.....
	6, 494	8, 616	8, 336	5, 560	5, 431	8, 150	.....	.....	.....
Average	6, 294	7, 632	8, 632	5, 928	5. 530	7, 239	.....	.....	.....

Discussion

In earlier work, an attempt has been made in this laboratory to eliminate the plasma clot from the tissue culture. Instead of embedding the cells in a plasma matrix, they have been allowed to adhere to the surface of a cellophane membrane (*8*) or to a glass surface such as the inner surface of the culture vessel (*9*) or the surfaces of a mass of helices (*10*).

In the study of the behavior of the cells in the mass of glass helices, the experimental data obtained suggested that the rate of circulation of the culture medium was one of the critical factors controlling the proliferation of the cells. In the present paper, an attempt has been made to analyze further this influence of the velocity of flow of the medium on the proliferation of the cells on a surface substrate. In order to obtain a simpler group of experimental conditions than those prevailing in three-dimensional substrate cultures, it has been convenient to use roller-tube cultures, in which the rate of flow of the fluid medium over the cells adhering to the inner surface of the roller tube has been controlled by the rate of rotation of the roller-tube drum.

Text-figure 2 shows that rate of increase of population in all cultures was maximal during the first week and progressively lessened during the second and third weeks. By the end of the first week the cultures that showed the most rapid gain in population had already exceeded 5 million nuclei. The ensuing decrease in the rate of population rise at about 5 to 6 million nuclei is similar to that seen in previous studies with strain L cells in T-15 flasks. It is interpreted as indicating that beyond this level of population density conditions associated with cell crowding, such as inadequate oxygenation of the cells and exhaustion or toxification of the limited volume of the nutrient fluid, have acted to reduce the rate of population increase.

During the early growth of the culture under the experimental conditions studied, the T-15 flask does provide far more optimal conditions for population increase than does the static tube or the slowly rotating tube culture, and does provide conditions highly favorable for rapid increase in culture population. This observation tends to confirm the general impression developed in our laboratory over a period of years that best results with at least many cell types are obtained in starting cultures of low inoculum population if a static culture is used, while with very high levels of cell populations some type of circulation of the fluid nutrient gives superior results.

Since, in the static T-15 flask culture and the static tube culture, the volume of nutrient and the number of cells in the inoculum, the control of temperature, etc., were comparable, this superior behavior of the flask culture must of necessity have been associated with the geometry of the culture. In the static T-15 flask the cells settled out under the influence of gravity, and were fairly uniformly distributed over the approximately 15 square centimeters of the glass floor of the flask; in the static tube culture the area under the surface of the nutrient was also approximately 15 square centimeters, but as the cells settled out on the lower surface of

the tube, its curvature tended to concentrate the cells at the lowest part of the circumference so that they were less evenly distributed than in the T-15 flask culture.

Further, the cells in the T-15 flask were covered by a layer of fluid nutrient approximately 2 mm. thick; in the static tube culture the layer was not of uniform thickness but varied from a thin film at the edge of the layer to more than 5 mm. thick at the center directly overlying the area where the curvature of the tube had concentrated the greatest number of cells. It appears probable that this thicker layer of fluid substantially interfered with oxygenation of the cells concentrated beneath it, and reduced the rate of population rise.

No definite explanation can be offered as to why the tube culture rotating at 9.4 r.p.h. was not appreciably superior in its rate of population increase during the first week to that of the static tube culture. By the end of the second week, however, at which time the cell population of the culture was in excess of 5 million, the slowly rotating culture (9.4 r.p.h.) does show a somewhat greater cell population increase as compared with the static tube culture. This is presumably due to somewhat more effective washing of nutrient over the cell and more effective oxygenation of the cells. It would seem that even during the first week the cells of the slowly rotating tube culture must have superior oxygenation, and better flow of nutrient, and it would seem that this advantage should be of such magnitude that it would be indicated in the data by more rapid rate of population gain. The fact that no such advantage of rotation was measured during the first two weeks suggests that in the slowly rotating tube there are factors, as yet undefined, which do interfere with rate of population increase. The data indicate, however, that these retarding influences seem to be overcome or compensated when the velocity of tube rotation is increased to 600 r.p.h., possibly by even more favorable increase of cell aeration and circulation of fluid over the cell.

The data therefore indicate that with a densely populated glass-substrate culture, the velocity of flow of the medium is definitely a major factor in the rate of nuclear proliferation of the culture. It indicates that for glass-substrate cultures prepared from strain L suspension inocula of the size employed, within the range studied, velocity rates of somewhere between 300 and 1,200 r.p.h. appear to be optimal. Since the internal diameter of the culture tube is 22 millimeters, this corresponds to a circumferential flow of the fluid over the tube surface of approximately 2,074 to 8,294 centimeters per hour or from about 34.6 to 138.2 centimeters per minute, as compared with about 1 centimeter per minute for roller tubes operating at the slower velocity usually used.

Conditions in these rotating culture tubes are so complex as to make it impossible with our present limited experimental data to evaluate quantitatively the importance of many secondary factors, other than velocity of rotation, which may and almost surely do influence cell proliferation to a greater or lesser extent. For instance this optimal range of nutrient flow is probably influenced by factors altering the ability of the cells to maintain

an anchorage on the glass susbstrate, and by the surface tension or the viscosity of the nutrient fluid. Further, with motion other than a smooth rotation, factors such as wave or foam formation may present further complications. Until a better evaluation and control of the relative importance of certain of these variables can be obtained, it appears probable that such dynamic circulation cultures should be used with caution in accurate quantitative studies.

In all cultures of the present study, adhesion of the cells to the surface of the glass culture vessel was good, and the major proportion of the cells appeared to be adherent to the glass at any one time. There were, however, many cells which floated loose, at all velocities; at higher velocities even more appeared to be free. While the high cell-population levels attained by cultures rotating at from 300 to 1,200 r.p.h. indicated that no major retardation of proliferation by the cells floating free had occurred, the data obtained do not allow a conclusion as to whether or not the increase in the number of loose cells was favorable or injurious to the rapid increase in cell population.

Data obtained warrant no conclusion that all cells that can be made to proliferate in culture will show a comparable behavior within the range of rotation velocities studied; nor is there any reason to believe that the optimum rotation velocity for all cell types will be the same. Since the rate of rotation velocity of the tube does have such a significant influence on the rate of cell proliferation, it appears that the multiple-velocity-drum roller-tube unit is definitely useful in establishing optimal culture conditions for the growth of various cell types in surface substrate cultures grown from inocula of cell suspensions.

Summary and Conclusions

Growth of pure strain L cells, implanted as cell suspensions directly on the glass substrate, was studied in T-15 type flasks and in roller-tube cultures maintained at various velocities of tube rotation between 0 and 1,200 revolutions per hour. Cultures were grown in a mixture of 40 percent horse serum, 20 percent 1:1.25 chick-embryo extract, and 40 percent Earle's saline. Culture medium was renewed thrice weekly. Quantitative changes in culture population were followed during 3 weeks by nuclear enumeration.

Starting with inocula of approximately 480,000 cells in 3 ml. of nutrient, rates of increase in populations were maximal during the first week; during the second and third weeks they declined progressively for all cultures. This decline was interpreted as associated with overcrowding of the cultures, with probable inadequate oxygenation and exhaustion or toxification of the medium.

During the first week the maximum rates of increase in population were shown by the T-15 flask cultures; during the second and third weeks these rates dropped rapidly, probably due to conditions associated with cell crowding.

The results obtained indicate that the static T-15 type culture is extremely well suited for quantitative work under such experimental conditions that the cell population is not too dense. Under these conditions it appears far superior to either the static tube or slowly rotating tube culture.

During the first two weeks, of all cultures under study, the lowest rates of population increase were shown by the static tube and the 9.4 r.p.h. tube cultures. Rates for these two were indistinguishable from each other, and after one week the population levels of cultures of either type were less than half those of the T-15 type cultures.

With increased rates of rotation of the tube cultures, the rates of population increase also rose and at the one-week period reached a maximum at 600 r.p.h. The rate of population increase at this time was indistinguishable from that shown by the T-15 cultures. During the course of the entire 3 weeks, however, no one velocity of rotation from this 300 to 1,200 r.p.h. range could be definitely considered as optimal.

During the second and third weeks of the periods studied, the rapidly rotating tube cultures showed continued rates of population increase which exceeded those of the T-15 flask cultures. During this period the total number of cells in the rapidly rotating tube cultures also exceeded the numbers in the static flask cultures. The rapidly rotating tube culture therefore seems well suited for use with extremely high density populations, although for accurate quantitative work certain factors in its use are pointed out as needing further study.

At higher velocities of rotation, in particular, quite a few of the cells were free-floating in the medium at any one time, but no conclusion could be reached as to whether this was advantageous or retardative to the rate of cell proliferation.

Data obtained warrant no conclusion that all cell types would show a comparable behavior within the range of velocities studied. It does appear that multiple-velocity roller-tube units may be of particular value in establishing optimal culture conditions for the growth of various cell types in surface substrate cultures grown from inocula of cell suspensions.

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Determination of Peripheral Blood Pressure in Unanesthetized Mice During Microscopic Observation of Blood Vessels [1, 2]

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The transparent-chamber technique as adapted to mice (*2*) provides access to a thin layer of subcutaneous and muscular tissue, in which measurements of peripheral blood pressure and correlated circulatory reactions may be made in unanesthetized mice. Measurements and observations may be made on one or several selected vessels throughout the day or repeatedly for many days.

The apparatus to be described here has been used mainly with the transparent-chamber technique and is based upon the principle of the microcapillary tonometer of the type reported by Roy and Brown in 1880 (*3*), and used by Danzer and Hooker (*4*), McMaster and Hudack (*5*), and by Krogh, Turner, and Landis (*6*). This indirect method has been found useful in this laboratory in studies on the mechanism of chemical agents in producing necrosis in transplanted sarcomas (*7–9*).

Methods

The transparent-chamber technique has been described previously in detail (*2*). The chamber is inserted between the layers of a fold in the dorsal skin of the mouse (fig. 1) permitting observation with the microscope, using transmitted light, of the tissues within one layer of skin composed of subcutaneous and muscular tissue. When the apparatus to be described is applied to such a tissue one may occlude vessels during observation and thus obtain an index of relative blood pressures.

The apparatus (text-fig. 1) consists of a mercury sphygmomanometer system and air reservoir connected to a glass tube which carries air to a thin rubber membrane across the end. As indicated in cross section in text-figure 1, the mouse is supported on a transparent-chamber stage. A micromanipulator is used to bring the membrane into contact with the under surface of the skin beneath the transparent chamber. Pressure applied by squeezing the sphygmomanometer bulb results in an increase in air pressure in the system which is transmitted through the membrane to the tissue.

A special feature of the apparatus is the air delivery system consisting of a glass tube attached to a solid lucite cone (text-fig. 1). This unit,

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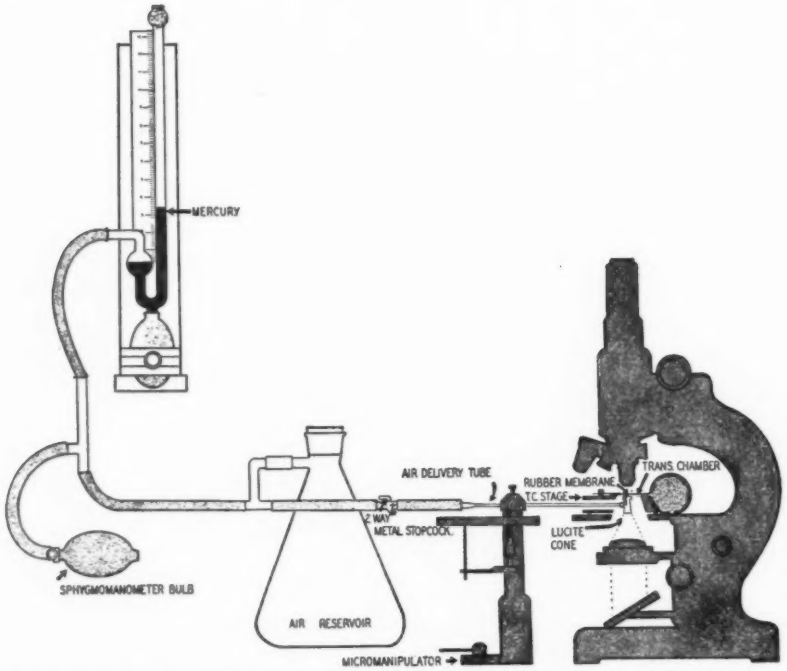
[2] A preliminary abstract has been published (*1*).

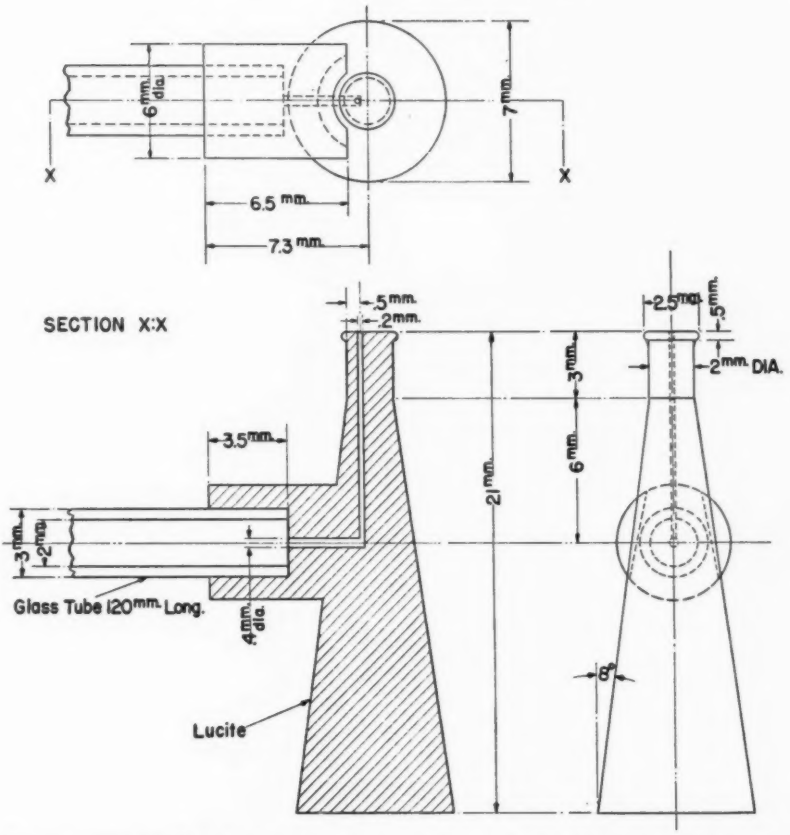
[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.



TEXT-FIGURE 1.—Arrangement of apparatus for the measurement of blood pressure in vessels within a transparent chamber in a skin fold of a mouse.

illustrated in detail in text-figure 2, provides for transmission of light and air pressure. Light from a condenser of 16 mm. focal length is focused on the base of the lucite cone and transmitted through the translucent membrane and tissues to the microscope objective. As the air pressure is increased, the membrane is distended so as to cause occlusion of the blood vessels between the membrane and the cover-slip of the chamber. The tip of the lucite cone has a narrow ledge to facilitate tying of the membrane. Passage of air through the lucite cone is provided for by a small hole drilled eccentrically to avoid the production of a dark shadow in the center of the transilluminated microscope field.

The membrane must be tied across the tip of the lucite cone with sufficient slack to avoid errors due to the pressure required to overcome elasticity. This is accomplished as follows: A thin coating of Lubriseal is applied to the sides of the lucite cone, just above the air delivery tube. A piece of the membrane approximately one inch square is centered over the tip of the cone, and a washer is brought down over the membrane in order to bring it into contact with the sides of the lucite cone. Air is passed through the system at a pressure sufficiently high to cause bulging of the membrane during the procedure of tying with waxed thread. The thread is passed around the lucite cone, between the tip and the washer. If necessary to create the desired flexibility, the tied membrane may be



TEXT-FIGURE 2.—Detail of air delivery system, arranged with the membrane just beneath the epithelial surface of the skin within the transparent chamber.

slid upward toward the ledge at the tip of the lucite cone. Thus, the membrane is flaccid at zero pressure, and when placed beneath the tissue as described below, is not really stretched at any time during measurement.

The stage to support the chamber in position, described in detail in a previous publication (*2*), has been modified by raising the platform for support of the transparent chamber and skin fold to provide a space 8 mm. high for introduction of the lucite cone between the platform and its base. As shown in text-figure 1, this permits placing the tip of the lucite cone directly beneath the epithelial surface of the tissue within the chamber.

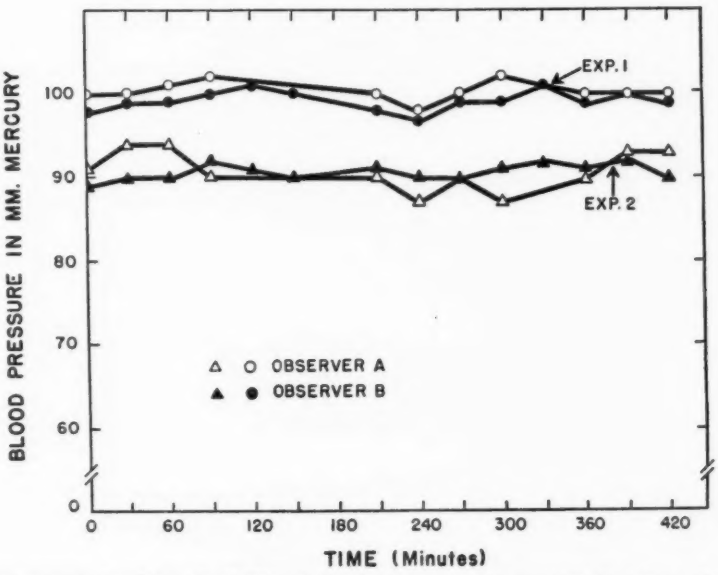
Procedure for Blood-Pressure Measurement

Before the mouse is placed in position, the pressure is raised slightly in order to deflect the membrane covering the tip of the lucite cone. The highest point of the membrane is centered in the microscope field and the

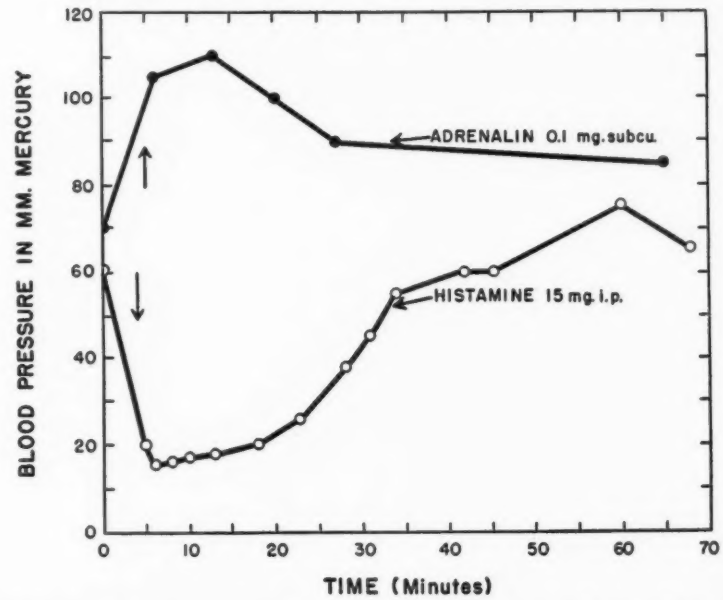
condenser focused on the base of the lucite cone to give maximum illumination. The mouse is then placed in its holder, the transparent chamber is mounted on the stage, and the area or vessel of interest is selected. The micromanipulator is used to move the lucite cone vertically, bringing the membrane into contact with the lower surface of the tissue beneath the vessel. This adjustment is made during examination through the microscope, while the membrane is under a pressure of 10 mm. of mercury in order to obtain a slightly convex surface of contact of the membrane with the epithelial surface of the skin, and thus insure uniform occlusion at the center of the microscope field. The pressure is then dropped to zero and the micromanipulator is adjusted to insure contact between the membrane and the tissue. As the pressure is raised, the vessel narrows and then becomes occluded, stopping the flow of blood. The blood pressure is taken at the point at which forward blood flow is resumed as the pressure is lowered after occlusion. In measurements upon arteries, the pulse wave is visible at the proximal side of the occluded segment of the artery, appearing at maximum amplitude approximately midway between the pressure required for complete occlusion and that obtained when the original caliber of the vessel has been restored. Figure 2 shows the brightly illuminated area over the membrane, a vein occluded by increased pressure, and an adjacent patent artery. Note also the absence of interference to blood flow in the surrounding vessels.

The small diameter of the lucite tip (2.5 mm.) as compared with that of the chamber (14 mm.) permits localization of pressure to a limited area of tissue. Thus, it is possible to scan the entire chamber area and to measure the pressure of an artery or vein, or of branches leading to or from the capillary bed as illustrated in figure 3. Examples of such measurements are given in table 1, where pressure determinations are recorded for the main arteries within the chamber, their successively smaller branches, capillaries, venules, and veins. These records show a direct correlation between blood pressure and vessel size, and except for discrepancies in venule and venous pressures, to be discussed later, are in accord with the direction of blood flow. The effects of hypotension on peripheral blood pressure are shown in experiments 7 and 9 of table 1, and the effects of hypertension in experiment 8. These determinations show that the decreased values obtained in passing from larger to smaller vessels are not due simply to lesser diameter, but reflect real pressure changes. In this procedure, the results obtained are considered as relative rather than absolute, and hence are of value in detecting relative differences in pressure between vessels of varying diameter as indicated in table 1, or changes occurring within a single vessel after various stimuli, as recorded in table 2.

Text-figure 3 shows the reproducibility of blood-pressure measurements in two experiments made independently by two observers over a 7-hour period. The blood-pressure response to adrenalin and to histamine, and the recovery after injection of each of these substances, are shown in text-figure 4.



TEXT-FIGURE 3.—Reproducibility of blood-pressure measurements in artery of 0.04 mm. diameter.



TEXT-FIGURE 4.—Blood-pressure responses to adrenalin and to histamine in artery of 0.04 mm. diameter.

TABLE 1.—*Determinations of blood pressure and vessel diameter in peripheral blood vessels during control period, and as affected by hypotensive or hypertensive procedures*

Experiment No.	Experimental procedure	Reading*	Arterial branches				Arteriole		Capillary 1	Capillary 2	Venous capillary	Venule 1	Vein 2
			1	2	3	4	1	2					
1	Control	B.P.	95	95	90	60	35	.....	25	20	.....	.....	.....
		Diam.	.07	.08	.07	.03	.005	.....	.005	.006	.....	.....	.....
2	Control	B.P.	80	65	45	30	28	.....	25	22	17	18	8
		Diam.	.06	.05	.02	.01	.005	.....	.006	.006	.01	.02	.04
3	Control	B.P.	65	55	45	40	30	.....	27	.....	10	18	.....
		Diam.	.07	.03	.03	.01	.....	.....	.005	.....	.005	.06	.....
4	Control	B.P.	87	.....	.....	55	39	.....	25	.....	.....	22	33
		Diam.	.06	.....	.....	.01	.006	.....	.006	.....	.....	.01	.04
5	Control	B.P.	60	.....	.....	39	33	33	22	.....	.....	22	.....
		Diam.	.06	.....	.....	.01	.005	.005	.006	.....	.....	.02	.....
6	Control	B.P.	97	75	60	.....	.....	.....	.....	.....	.....	.....	.....
		Diam.	.055	.04	.02	.....	.....	.....	.....	.....	.....	.....	.....
7	Control period	B.P.	82	60	46	33	26	.....	15	.....	11	18	25
		Diam.	.05	.03	.03	.02	.....	.....	.....	.....	.01	.04	.05
	After accidental dehydration and starvation—16 hrs.	B.P.	35	27	25	20	12	.....	.....	.....	.....	4	.....
		Diam.	.07	.04	.04	.01	.005	.....	.....	.....	.....	.03	.....
8	Control period	B.P.	60	50	35	30	30	.....	.....	.....	.....	.....	.....
		Diam.	.07	.04	.03	.03	.005	.....	.....	.....	.....	.....	.....
	Adrenalin, 100 micrograms	B.P.	90	70	45	35	.....	.....	.....	.....	.....	.....	.....
		Diam.	.07	.05	.04	.03	.....	.....	.....	.....	.....	.....	.....
9	Control period	B.P.	95	90	90	45	25	.....	.....	.....	.....	.....	.....
		Diam.	.06	.06	.06	.01	.005	.....	.....	.....	.....	.....	.....
	10 mg. histamine (intraperitoneally)	B.P.	40	35	25	20	15	.....	.....	.....	.....	.....	.....
		Diam.	.06	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	Recovery (next day)	B.P.	90	85	80	42	22	.....	.....	.....	.....	.....	.....
		Diam.	.05	.....	.04	.....	.005	.....	.....	.....	.....	.....	.....

*Blood pressure is expressed in millimeters of mercury, and vessel diameter in millimeters.

TABLE 2.—*Blood-pressure changes within a single artery following parenteral injection of various agents*

Exp. No.	Procedure	Time in minutes								
		0	10	20	30	40	50	60	70	80
1	Adrenalin intraperitoneal (200 μ)	55	110	110	110	.....	.....	.....	70	70
2	Adrenalin intraperitoneal (200 μ)	110	129	140	140	135	126	116	105	109
3	Histamine phosphate intraperitoneal (18 μ)	85	40	43	82	.....	.....	.....	.....	.....
4	Histamine phosphate subcutaneous (100 μ)	110	65	75	.....	110	110	.....	.....	.....

Exp. No.	Procedure	Time in hours													
		0	½	1	1½	2	2½	3	3½	4	4½	5	5½	6	24	48
5	Bacterial polysaccharide (135 μ) subcutaneous	100	.....	.....	.....	.....	50	.....	.....	60	.....	.....	.....	60	.....	.....
6	Bacterial polysaccharide (360 μ) subcutaneous	75	.....	.....	45	51	51	.....	.....	.....	51	.....	.....	.....	35	85
7	Podophyllotoxin (100 μ) subcutaneous	55	.....	45	.....	40	.....	.....	.....	30	.....	.....	.....	42	55	.....
8	Podophyllotoxin (200 μ) subcutaneous	70	.....	50	.....	45	.....	.....	.....	.....	.....	.....	.....	20	60	70

Discussion

The application of this indirect method to the transparent-chamber technique makes possible microscopic observation of blood vessels combined with simultaneous determinations of relative arterial pressure. Measurements are not limited to vessels in subcutaneous tissue, but may be made as well in the panniculus layer of striated muscle. The method provides visual objective criteria of relative pressure gradients in arteries, arterioles, and capillaries in unanesthetized mice, and permits return to the same vessels over a period of many days. With minor modifications it is readily adaptable to other sites, such as the ear, or organs of other animals.

The indirect method for blood-pressure measurement described herein minimizes certain sources of error discussed by Krogh (*10*), who has pointed out that accuracy of indirect blood-pressure determination is enhanced if the area under pressure is clearly defined; the kind of vessel on which determinations are made is exactly defined; and measurements are made over areas where the skin is supported. Various other indirect methods have been described for blood-pressure measurement in mice which do not involve observation with the microscope of the vessel within which pressure is to be determined. These methods will be omitted from this discussion.

As an additional source of error, Landis (*11*) has mentioned the possible effect of total obstruction of flow. In this procedure, however, pressure is determined the instant blood resumes forward flow after occlusion. For increased accuracy one may consider, as Landis points out (*11*), that pressure measurements upon a given vessel refer not to the occluded vessel, but to the next proximal branch. Thus, one measures the pressure of the stationary column of blood in the occluded vessel, as an indication of the lateral pressure exerted by the flowing blood in the unobstructed branches. A third source of error depends upon differences in vessel diameter. Thus, as shown in table 1, the values obtained in measurements of a large vein may be 5 to 10 mm. of mercury greater than those obtained in a small venule draining into it. The magnitude of this error is directly correlated with the diameter of the vein and appears to be unavoidable. For these reasons the measurements obtained are considered as relative rather than absolute.

It should be recognized that measurements of blood pressure by an indirect method of this type are influenced both by the tone of the vessel and by the hemodynamic pressure. However, the relative values obtained by this method, as given in table 2, are supported by visual evidence of decreased rates of flow during hypotension, as indicated by the change from linear or streamlined flow to a granular type of flow in which the cellular elements become visible. Therefore it does not seem probable that alterations in vasomotor tone alone could account for the reduction in pressure required for the occlusion of the vessel by this indirect method.

Summary

A method utilizing a membrane manometer system is described for indirect measurement of peripheral blood pressure in vessels within transparent chambers in mice. Measurements are made in unanesthetized mice at intervals throughout the duration of the preparation, approximately 30 to 60 days. As the field is visualized under the microscope at magnifications up to 200 ×, one can obtain a measure of arterial systolic pressure and of arteriolar, capillary, and venous pressures. The blood-pressure measurements may be correlated with observations or measurements of other circulatory phenomena, such as rates of flow and arteriolar vasomotion. The measurements obtained are considered as relative rather than absolute.

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PLATE 64

FIGURE 1.—Transparent chamber in skin fold of mouse, showing the vascular bed accessible to measurement of blood pressure.

FIGURE 2.—Brightly illuminated area of tissue over membrane during occlusion of vein. × 50

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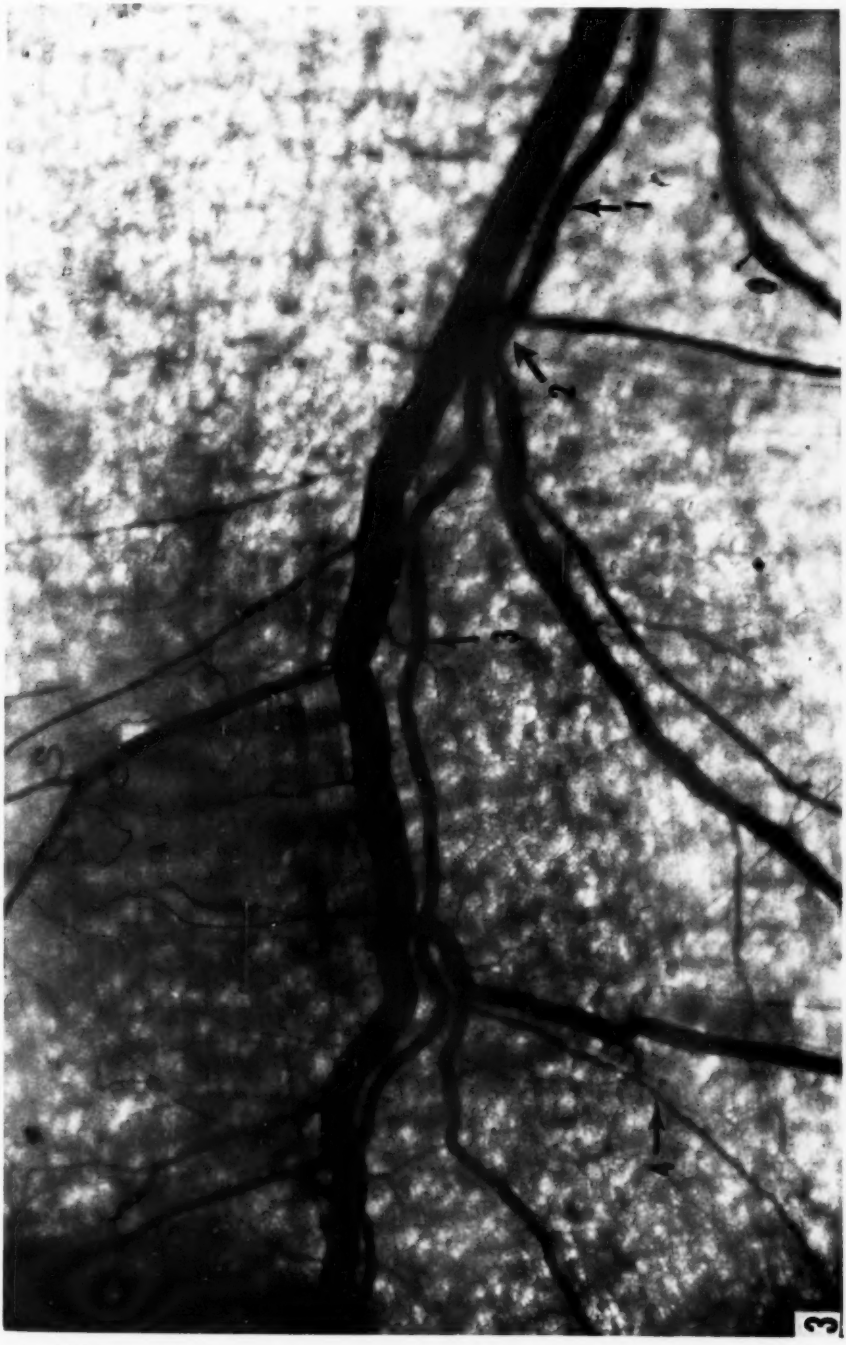
277342—54——17

PLATE 65

FIGURE 3.—Vascular bed of tissue within transparent chamber. Arterial branches correspond, as indicated, to the determinations given in table 1, experiment 1. × 25



Vascular Reactions of Normal and Malignant Tissues *in Vivo*. VI. The Role of Hypotension in the Action of Components of Podophyllin on Transplanted Sarcomas [1]

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Podophyllin and certain of its components have been shown to have a colchicine-like action following topical application to skin (*1*, *2*). These components have also been reported to damage transplanted tumors in mice following subcutaneous injection (*3*, *4*). Ormsbee, Cornman and Berger (*5*) stated that podophyllin damaged tumor cells *in vitro*, but the *in vivo* mode of action of these compounds on tumor cells has remained obscure. In this paper, evidence is presented that the *in vivo* action of podophyllotoxin and of podophyllin in causing necrosis in transplanted sarcomas results indirectly from its hypotensive effects on the host.

Methods

The transparent-chamber technique was used as previously described (*6*) to obtain well-vascularized growing sarcomas for *in vivo* microscopic study. The tumors used were strain L sarcoma and Sarcoma 37, both carried in transplant at this Institute. A determination of arterial systolic pressure was obtained in unanesthetized mice by an indirect method (*7*) in which an artery adjacent to the tumor growing within the chamber area was observed microscopically. In this procedure the artery is occluded by means of air pressure transmitted through a flexible membrane. The blood-pressure changes were correlated with observations on blood flow in the tumor and surrounding normal tissues within the chamber area. These were supplemented by photomicrographic records, and by measurements of the percentage of tissue occupied by functional blood vessels,[3] using methods previously described (*8*, *9*).

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[1] Received for publication April 22, 1953.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

[3] Vascular percentage: This index is a relative value applicable as an approximation to successive changes in vascularity of tissue. In this method a microscope fitted with an eyepiece arranged to show a fixed pattern of 4 points in the focal plane is used. For any one count this pattern is presented to the field in view 100 times and the percentage of visual contacts made with functioning vessels is recorded. A point is considered as a contact when the vessel is sufficiently in focus so that movement of blood within it can be detected.

277842—54——18

In addition to the above circulatory changes, other *in vivo* indices of tumor damage were used as described in detail previously (*10*): vascular stasis and hemorrhage within the tumor, the later development of grayish opacity of such avascular areas of tissue in contrast to the more translucent appearance of unaffected areas, failure to reestablish circulation in affected areas, liquefaction of previously opaque areas, temporary slowing of growth rate, and ulceration of skin over the protruding tumor mass. These criteria were supplemented by histologic study of tissues fixed in Zenker-formol, sectioned, and stained with hematoxylin and eosin.

Observations and quantitative measurements were made as indicated above, prior to and at frequent intervals following injection of podophyllin, podophyllotoxin, or picropodophyllin.

In addition to the above experiments using the transparent chamber, observations of blood flow and indirect measurements of blood pressure were made in the peripheral arteries in ears of non-tumor-bearing mice, following injection of podophyllotoxin and a derivative. The mice were not anesthetized, but were adequately immobilized by placing them in a mouse holder with the head restrained by a plastic cone. A film of immersion oil was placed between the ear and a cover-slip. Blood-pressure measurements were made using the air delivery system previously described (*7*). Both the air delivery system and the cover-slip were controlled by micromanipulators.

Stock preparations of podophyllin, podophyllotoxin, and picropodophyllin [4] were weighed out in small quantities and dissolved or dispersed by grinding in a small volume of olive oil. The mixture was warmed moderately in a water bath to hasten this process. The small volume was then diluted with additional olive oil to make the appropriate dilution. Preparations were made and used the same day. Acetylpodophyllotoxin-ω-pyridium chloride, hereafter designated as APPC, was dissolved in distilled water and used immediately.

Results

Podophyllin

The results of subcutaneous injection of podophyllin are given in table 1, experiments 1 to 4. Slowing of rates of blood flow in the arteries, veins, and capillaries of the normal tissue, as well as in those of the tumor, occurred within the first hour after injection. This was followed by occlusion or stasis of flow in many vessels. Tumor ischemia, as indicated by determinations of the percent of functional vessels, became apparent within 1 or 2 hours and was most pronounced after 5 or 6 hours.

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[4] The author wishes to thank Dr. M. J. Shear and Dr. Joseph Leiter, National Cancer Institute, for the materials used and suggestions made on dosages. The following information was provided:

Podophyllotoxin—maximum tolerated dose 30 micrograms per gram of body weight; minimal effective dose, 2 micrograms per gram.

Acetylpodophyllotoxin-ω-pyridinium chloride—maximum tolerated dose, 80 micrograms per gram; minimal effective dose, 5 to 8 micrograms per gram.

TABLE 1.—*The effect of subcutaneous injection of podophyllin, podophyllotoxin, and picropodophyllin on peripheral blood pressure, blood flow, and extent of tumor destruction*

Exp. No.	Strain	Agent	Dose (μg.)	Tissue	B. P. and V.P.*	Hours after injection								Days after injection †					Remarks
						0	1	2	3	4	5	6	7	1	2	3	4	5	
1	C3H....	Podophyllin.....	40	Sarcoma L.....	V. P.	47	....	36	0	0	0	0	0	0	0	....	(‡)	....	Sluggish circulation in tumor and in adjacent arteries and veins. Hemorrhage after 5 hours of vascular stasis. Tumor opaque next day.
2	CAF_1...	Podophyllin.....	575	Sarcoma 37.....	V.P.	43	46	....	33	....	....	7	....	0	(§)	....	....	....	Complete stasis of tumor circulation within 6 hours; avascular and with hemorrhage at periphery after 1 day.
3	CAF_1...	Podophyllin.....	1,000	Sarcoma 37.....	V.P.	39	46	....	....	36	....	3	....	(‡)	....	....	....	....	Occlusion and stasis of tumor vessels after 6 hours.
4	C3H....	Podophyllin.....	1,300	Striated muscle.....	V.P.	41	41	43	....	39	18	30	....	33	38	(‡)	....	....	Decrease in capillary circulation in muscle after 5 hours, partial recovery next day.
5	CAF_1...	Podophyllotoxin..	100	Sarcoma 37.....	V.P.	61	....	....	1	....	....	....	....	....	(§)	....	....	....	Complete vascular stasis except for one vessel after 3 hours, followed by central opacity and necrosis.
6	C3H	Podophyllotoxin..	100	Main artery.....	B.P.	55	45	40	35	30	30	40	....	55	....	....	....	....	Slowing of blood flow correlated with decreased blood pressure.
7	C3H....	Podophyllotoxin..	150	Main artery.....	B.P.	72	52	....	55	68	....	71	....	....	....	....	....	....	Slowing of blood flow correlated with decreased blood pressure.
8	C3H....	Podophyllotoxin..	200	Artery supplying tumor‖.	B.P.	70	50	45	....	....	25	20	....	60	....	....	....	....	Sluggish circulation 2 hours after injection. Almost complete stasis after 5 hours.
				Sarcoma L.....	V.P.	42	43	38	....	....	....	1	....	7	....	....	33	....	Petechial hemorrhage throughout tumor in 6 hours. Next day central opacity of tumor. Two days later, recovery of tumor circulation around central area of necrosis.
		Podophyllotoxin (5 days later)	250	Sarcoma L.....	V.P.	40	41	27	....	4	2	....	....	0	(§)	....	....	....	Sluggish circulation apparent within 3 hours. Complete tumor ischemia within 24 hours.
9	C	Podophyllotoxin..	250	Main artery.....	B.P.	72	75	35	30	40	35	44	32	52	65	....	....	80	Sluggish circulation after 2 hours. Recovery of circulation in muscle after 24 hours.
				Striated muscle.....	V.P.	40	....	....	30	....	....	....	28	34	....	....	....	....	
10	CAF_1...	Podophyllotoxin..	260	Artery supplying tumor‖.	B.P.	120	95	70	75	....	90	....	50	....	....	....	....	....	Sluggish circulation after 2 hours. Hemorrhage and opacity with complete tumor ischemia 1 day later.
				Sarcoma 37.....	V.P.	34	....	....	....	....	33	....	19	0	....	....	....	....	
11	CAF_1...	Podophyllotoxin..	280	Artery supplying tumor‖.	B.P.	75	65	40	38	43	....	43	....	75	85	....	....	....	Slow circulation, hemorrhage in tumor after 2 hours. Circulatory stasis and hemorrhage after 1 day. Central necrosis after 24 days.
				Sarcoma 37.....	V.P.	34	....	....	23	....	....	....	8	16	25	....	....	....	
12	C3H....	Podophyllotoxin..	280	Sarcoma L.....	V.P.	29	11	....	....	....	0	....	....	....	....	....	....	....	Functional vessels only at periphery of tumor after 1 hour. Complete circulatory stasis after 5 hours.
13	C3H....	Picropodophyllin..	280	Artery supplying tumor‖.	B.P.	85	90	95	87	80	....	....	....	....	....	....	....	....	No significant change in tumor or host circulation. No tumor damage.
				Sarcoma L.....	V.P.	38	....	....	37	33	....	....	....	....	....	....	....	....	
14	C.....	Picropodophyllin..	280	Main artery.....	B.P.	82	80	88	....	83	....	....	....	....	....	....	....	....	No significant change.

*Blood pressure and vascular percent are expressed as B.P. and V.P. throughout the table. The blood pressure at "0" time was taken immediately prior to injection. Differences in blood pressure at the start of each experiment represent normal variation among the mice and selections for pressure measurement of arteries of different sizes. Vascular percent represents functional vessels only.

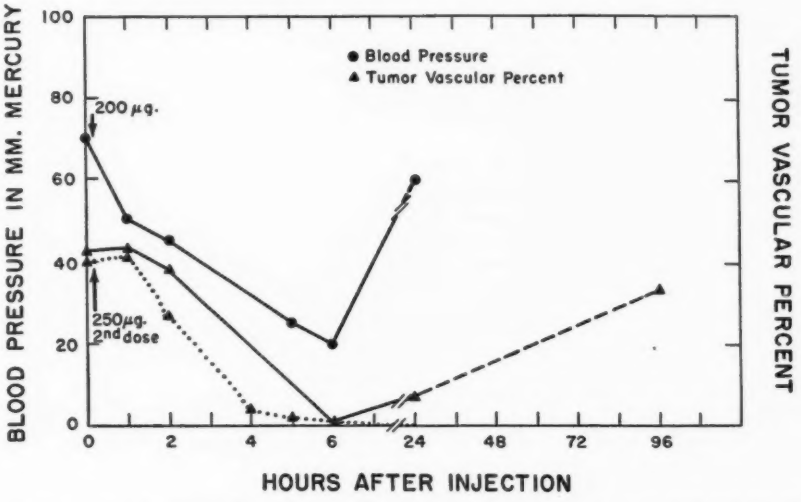
†All animals recovered unless otherwise indicated. ‡Died. §Sacrificed for section. ‖Located in adjacent tissue.

Tumor damage, as indicated by hemorrhage or decreased translucency, was first noted within 24 hours, and developed in approximately 48 hours as grayish opacity or liquefaction necrosis. The dosages used were lethal to 3 of 4 mice used after 1, 3, or 4 days, respectively. One mouse was sacrificed to obtain histologic sections of the tumor. This section (experiment 2, table 1) confirmed the *in vivo* interpretation of diffuse necrosis. Figure 1 shows the rich blood supply of a sarcoma prior to injection of podophyllin (table 1, experiment 2). Circulatory stasis and occlusion developed rapidly over a period of several hours after injection of podophyllin, leading to almost total ischemia at the end of 6 hours. Figure 2 shows total collapse of the vascular bed and general opacity of the same tumor as it appeared 24 hours after injection. These circulatory effects of podophyllin were identical to those seen after injection of various agents that lowered peripheral blood pressure (*7*, *11*, *12*), and hypotension is inferred to have occurred after injection of this agent. However, the highly toxic properties of the crude podophyllin made it desirable to extend these observations to podophyllotoxin, a less toxic derivative of known chemical constitution.

Podophyllotoxin

Injection of podophyllotoxin resulted in the development of peripheral hypotension, accompanied by arterial dilatation, sluggish blood flow, apparent hemoconcentration and increased circulating leukocytes, and cessation of arteriolar vasomotion and of striated muscle contraction. Capillaries became occluded or showed stasis of blood flow in both striated muscle and tumor tissue. The results of 8 experiments using the transparent-chamber technique are summarized in table 1 (experiments 5 through 12). In five similar experiments, records made by means of time-acceleration cinephotomicroscopy gave confirmatory results. It is apparent from table 1 that hypotension occurred in mice with or without transplanted tumors. The decrease in vascular percentage of the tumors, and slowing of circulation in normal and tumor tissue was coincident with the onset of hypotension. Hypotension became apparent within ½ to 1 hour after injection, while *in vivo* evidence of tumor damage as manifested by stasis, hemorrhage, and decreased translucency occurred only as a result of several hours or more of low blood pressure and ischemia, with necrosis as the end result. In these experiments as in those previously described (*11*, *12*), blood flow in capillaries of striated muscle decreased as a result of hypotension, but the vessels were not irreversibly damaged, and circulatory rates returned to normal as the blood pressure returned to normal levels. Blood pressure rose toward preinjection levels within 1 day. Simultaneously tumor circulation returned to normal levels in uninvolved areas and growth was resumed.

The results of experiment 8 of table 1 are illustrated in detail in text-figure 1 and figures 3 to 6. Figure 3 shows the hemorrhagic appearance of the tumor 6 hours after the first injection of podophyllotoxin. At this time, as indicated in the graph (text-fig. 1) the blood pressure had dropped



TEXT-FIGURE 1.—The effect of two injections of podophyllotoxin on peripheral blood pressure and percentage of functional capillaries within the tumor. The dotted line indicates results of a second dose on tumor vascular level. Blood-pressure measurements could not be taken after the second dose, due to increased size of tumor.

from 70 to 20 mm. of mercury, and the percentage of functional capillaries within the tumor from 43 to 1. Figure 4 shows the appearance of this tumor 24 hours after injection, with central opacity, residual hemorrhage, and partial recovery of circulation at the periphery. At this time, as shown in text-figure 1, the blood pressure had returned to 60 mm. of mercury, and the percentage of functional vessels had increased to 7. Three days after the first injection, as illustrated in figure 5, the tumor showed central necrosis, with recovery of circulation in the peripheral areas.

A second injection of 250 micrograms of podophyllotoxin was given 5 days after the first dose, when the vascular percentage in the involved areas had returned to 40 (text-fig. 1). Decreased rate of circulation within the tumor capillaries was apparent within 1 hour. Functional capillaries decreased to 2 percent, 6 hours after injection, and the tumor became avascular with hemorrhage at the periphery. Figure 6 shows a small zone of vascularized tumor tissue at the edge of the larger opaque and hemorrhagic tumor mass, as it appeared 24 hours after the second injection.

The experiments presented in table 2 provide evidence that the decreased rates of flow and the drop of blood pressure expressed a general effect on the peripheral circulation and were not restricted to the area of tissue included within the transparent chamber. Experiments 1 through 3 indicate the level of blood pressure in control mice over a period of 4 to 6 hours. Experiments 9 through 12 illustrate the considerable blood-pressure fall after dosages of 200 to 275 micrograms (approximately 8 to 10 micrograms per gram of body weight), with return to normal levels

TABLE 2.—*The effect of subcutaneous injection of components of podophyllin on arterial blood pressure as measured in ears of strain C mice*

Exp. No.	Agent	Mouse weight (*gm.*)	Dose		Blood pressure—hours after injection								
			(*μg.*)	(*μg./gm.*)	0	1	2	3	4	5	6	7	—24
1	Isotonic saline		0. 2 cc.		65	65	62	65	60	68			
2	"				73	75	–	72	75	75	73		
3	Isotonic saline	26	0. 2 cc.		82	85	78	76	80	–	–		
4	Podophyllotoxin	25	50	2	80	75	70	–	–	–	–		
5	Acetylpodophyllotoxin-ω-pyridium chloride	26	65	2. 5	80	80	78	74	66	66	64	65	80
6	"	28	70	2. 5	70	62	55	53	50	–	60	–	70
7	"	29	145	5	75	72	58	53	53	50	68	72	– –
8	"	27	135	5	73	58	50	38	33	35	–	62	
9	Podophyllotoxin		200		80	55	–	30	30	25	30	–	65
10	"		200		75	68	–	40	35	25	32	–	80
11	Acetylpodophyllotoxin-ω-pyridium chloride	26	260	10	75	68	55	42	38	30	30	35	70
12	"	28	275	10	75	72	50	49	55	55	45	55	80

by the next day. Dosages at or below the minimal effective dose[4] (experiments 4 through 8) resulted in less severe blood-pressure fall, with return toward control levels usually in 6 or 7 hours.

Picropodophyllin

Picropodophyllin was selected for study as an alkali conversion product of podophyllotoxin which had been found to be without effect on transplanted sarcomas at the doses used.[4] The results of these experiments are shown in table 1, experiments 13 and 14. The results were completely negative, with neither circulatory effects nor evidence of tumor damage.

Discussion

Considerable evidence has been obtained by various workers that podophyllin or podophyllotoxin produce mitotic arrest when applied topically to skin (*1*, *2*); inhibit mitosis in tissue culture (*5*); or act as a mitotic poison at metaphase, when injected intraperitoneally into rats bearing sarcomas in the ascitic form (*13*). Nevertheless, it cannot be assumed without additional evidence, that mitotic inhibition is either the sole or the primary mode of action of these agents in causing partial necrosis of solid vascularized tumors in the living animal when injected at a site distant from the tumor.

The experiments reported here provide evidence that circulatory effects of the agents, which act primarily on the host and only secondarily on the tumor, seem sufficient to account for their major damaging action on transplanted sarcomas. Observations of blood flow and determinations of blood pressure in ears of non-tumor-bearing mice show that the circulatory effects are not limited to vessels within the transparent chamber, or to vessels adjacent to growing tumors. It has been shown in these experiments, as well as in previous work (*11*, *12*), that circulatory slowing associated with hypotension results in severe restriction of blood flow through transplanted sarcomas; and that transplanted sarcomas are quite susceptible to prolonged ischemia (*10*). Cameron (*14*) has stated in this connection that of the numerous causes of cell death, including ischemia, all give the same sequence of cell change. Thus, any direct cytotoxic properties that these agents possess would be masked by their above-described action in decreasing blood flow through various tissues. Studies reporting cytologic evidence of early cell damage after injection of these agents (*4*) do not resolve the question of their direct cytotoxic action *in vivo*, since ischemia, also, has been shown to result in cloudy swelling, decreased transparency of cells, and hyperchromatosis, pyknosis, or karyorrhexis. Within susceptible tissues of the central nervous system, degenerative changes may occur after 10 to 15 minutes of ischemia [see review by Cameron (*14*)].

In studies on the mechanism of action of substances that induce damage in transplanted sarcomas, it would appear important to rule out circula-

[4] See footnote 4, page 880.

tory effects on the host before it is possible to ascertain whether a given substance has a direct action *in vivo* on a vascularized solid tumor. A recently described "diffusion-type" modification of the transparent-chamber technique (*15*) would seem to be applicable to these problems. In this system a fragment of tissue may be implanted as a graft and yet be kept completely separated from the cells and blood supply of the host by means of a sheet of a filter material made from cellulose derivatives. Cellular behavior may be accurately observed or photographed under conditions such as to constitute a literal "tissue culture *in vivo*."

Summary

The possible role of hypotension in tumor damage induced by components of podophyllin was investigated, using the transparent-chamber technique and an indirect method for measurement of peripheral blood pressure. *In vivo* microscopic observations on blood flow in sarcomas and surrounding normal tissues, or in ears of non-tumor-bearing mice, were made prior to and following subcutaneous injection of podophyllotoxin at varying dosages. Measurements of changes in the percentage of functional tumor capillaries were correlated either with determinations of systolic blood pressure or with observations of blood flow in arteries of normal tissue adjacent to tumors growing within the transparent chambers. Similar determinations were made following subcutaneous injection of picropodophyllin.

Evidence was obtained that the hypotensive effects of podophyllin and of podophyllotoxin on the peripheral circulation of the host seem to be sufficient to account for the observed tumor damage following injection of these substances.

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PLATE 66

FIGURE 1.—Sarcoma 37 prior to injection of 575 micrograms of podophyllin, showing rich capillary blood supply, with vascular percentage of 43. Table 1, experiment 2. × 25

FIGURE 2.—Same tumor as figure 1, 24 hours after injection of podophyllin, showing tumor ischemia and opacity. × 25

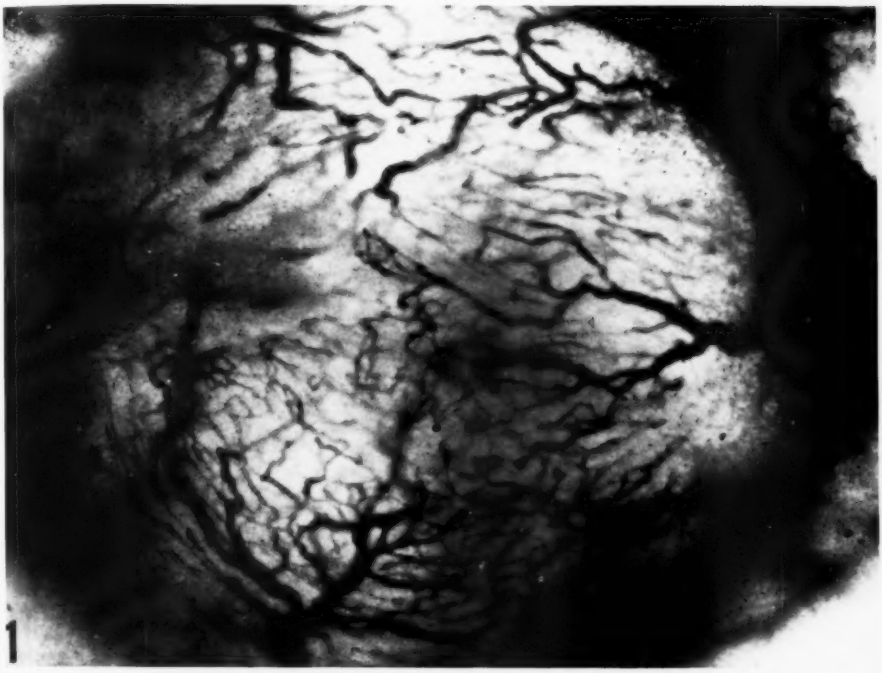
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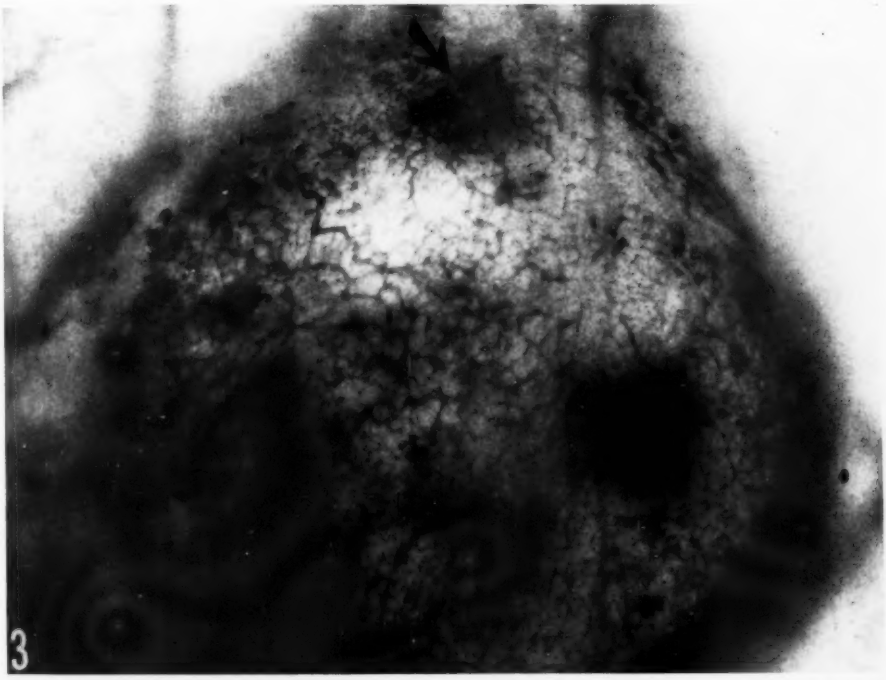
PLATE 67

(Figures 3 through 6 refer to experiment 8, table 1)

FIGURE 3.—Sarcoma L, 6 hours after injection of 200 micrograms of podophyllotoxin, showing hemorrhage throughout the tumor. × 18

FIGURE 4.—Sarcoma L, 24 hours after injection, showing central opacity, residual hemorrhage, and partial recovery of circulation at the periphery. × 18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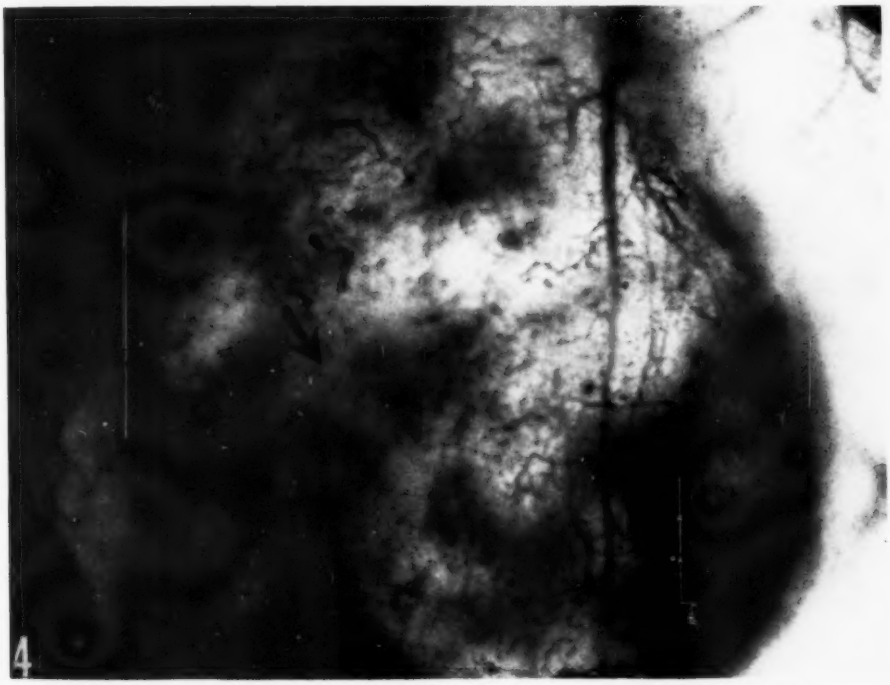
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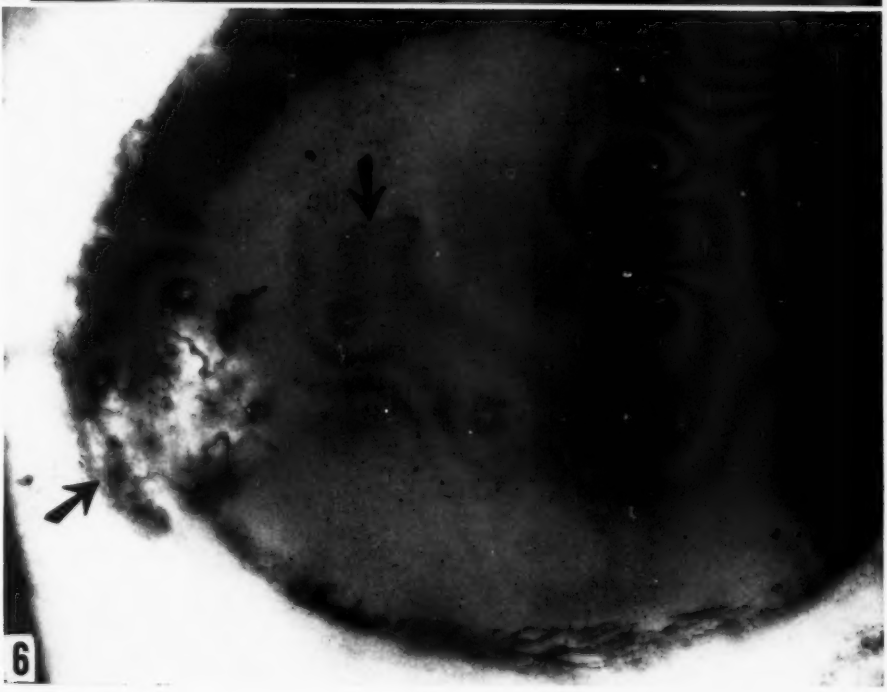
PLATE 68

FIGURE 5.—Sarcoma L, 3 days after injection, showing central necrosis, with recovery of circulation within the peripheral areas. × 18

FIGURE 6.—Sarcoma L, 24 hours after second injection (250 micrograms of podophyllotoxin). Generalized opacity and hemorrhage, with surviving tissue, at periphery indicated by arrow. × 18



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Factors Influencing Tumor Distribution Among the Mammary Glands of the Mouse [1]

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Pullinger reported that two of the five pairs of mammary glands in mice of the $RIII_b$ strain showed an unusual predilection to hyperplastic nodule formation (*1*). That report stimulated an investigation of the tumor distribution among the mammary glands in a variety of mouse strains. The authors are indebted to Dr. H. B. Andervont and Dr. W. E. Heston of the National Cancer Institute for their co-operation in making the records of their animal colonies available for this study.

Methods

Since the gland in which any given mammary tumor originated could not be accurately determined by gross inspection, the data available for this study gave only approximate tumor locations such as "right anterior" or "left inguinal," etc. However, the tumor locations were always described in sufficient detail to enable one to classify each tumor as occurring in either a pectoral or an inguinal gland. It is apparent that if the tumors were distributed evenly among the glands, the proportion of pectoral to inguinal tumors would be 6:4, since six of the ten mammary glands of the mouse are located pectorally. On the other hand, if the tumor distribution were uneven and were to correspond to the distribution of hyperplastic nodules reported by Pullinger, the ratio of pectoral to inguinal tumors would be almost 9:4. With these considerations in mind, the mammary tumor distributions between the anterior half and the posterior half of the body were investigated in the records of 3,612 tumors that occurred in the breeding female mice of the four inbred strains A/He, BALB/cAn, C3H/He, and $C3H_f$/He, and in (BALB/cAn$\times$ C3H/An)F_1 hybrid females.

Results and Discussion

The data for each of the four strains and the F_1 hybrid group are presented in table 1. Even casual examination of these data shows that

[1] Received for publication September 21, 1953.

[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

TABLE 1.—*Distribution of mammary gland tumors in mice of 4 inbred strains and an F_1 hybrid*

Group of mice	Milk agent	Anterior tumors	Posterior tumors	Anterior/ posterior tumor ratio	Average tumor age (all tumors) in months
(BALB/cAn × C3H/An) F_1	−	260	98	10.6/4	22. 9
$C3H_f$/He	−	170	73	9.3/4	20. 2
Totals	.....	430	171	10.0/4	.....
A/He	+	669	348	7.7/4	10. 8
BALB/cAn*	+	483	262	7.4/4	10. 5
C3H/He	+	745	504	5.9/4	8. 5
Totals	.....	1, 897	1, 114	7.3/4	.....

*This group of BALB/cAn mice contained the mammary tumor agent (2).

as far as tumor distribution ratios are concerned, those animals containing the mammary tumor agent constituted a fairly homogeneous class in which the tumor distribution among the glands was quite uniform. Those animals without the mammary tumor agent constituted another fairly homogeneous class in which the percentage of anteriorly occurring tumors was elevated. There was also a correlation between the average tumor age[3] and the percentage of anterior tumors. Detailed statistical analysis was required to determine the relative influences and interrelationships of tumor age, tumor morphology, presence or absence of the mammary tumor agent, and genetic differences.

Influence of the Milk Agent on Tumor Distribution

The most important data concerning the role of the mammary tumor agent were obtained by a comparison of the strain C3H/He with the strain $C3H_f$/He. Genetic differences between these strains were at a minimum[4] so that differences in tumor distribution and in average tumor ages must be presumed to have been due to the action of the mammary tumor agent, which was present in the C3H/He but lacking in the $C3H_f$/He strain. The strain C3H/He mice presented an almost perfect 6:4 ratio of pectoral to inguinal tumors, as would be expected with a uniform tumor distribution. The strain $C3H_f$/He, however, gave a ratio of 9.3:4 which is close to that anticipated from the distribution of hyperplastic nodules reported by Pullinger for a strain lacking the mammary tumor agent. This difference in

[3] Age of an animal when the tumor was first discovered. In those exceptional animals in which two or more tumors were found, each tumor was treated independently.

[4] Strain $C3H_f$/He was derived from strain C3H/He by removing a litter of strain C3H fetuses from their mother by cesarean section and allowing them to be foster-nursed by a female mouse of strain C57BL known to be free of the mammary tumor agent. In this manner mice of strain C3H/He lacking the mammary tumor agent were obtained, and from such mice strain $C3H_f$/He was established (3).

ratios between strain C3H/He and strain $C3H_f$/He is statistically highly significant ($P<.05$). The difference in average tumor ages is readily apparent by inspection, and is also highly significant. We may, therefore, conclude with reasonable certainty that the absence of the mammary tumor agent did tend to increase the relative number of anteriorly occurring tumors and the average tumor age, and that the differences noted between those strains with the mammary tumor agent and those without were at least in part a function of the mammary tumor agent rather than of fortuitous genetic differences.

Relationship of Tumor Morphology to Tumor Distribution

Heston *et al.* (*3*), Andervont (*4*), and Mühlbock (*5*) have reported that a relatively high percentage of tumors occurring in animals lacking the mammary tumor agent are of an atypical morphologic type. Therefore, the data were analyzed to determine whether the occurrence of these "atypical" tumors could have accounted for the increase in relative tumor incidence in the pectoral glands of mammary tumor agent-free mice.

The data concerning the distribution of "typical" and "atypical" tumors in the mammary tumor agent-free mice are presented in table 2.[5] Classification of tumors as "typical" or "atypical" was made according to the criteria of Dunn (*6*). Approximately three-quarters of the tumor slides reviewed had been classified personally by Dr. Dunn and those remaining were diagnosed by the present authors. Those cases in which no histologic section was available are recorded in the table under the heading "no slide available."

The distribution of "atypical" tumors in the combined groups of mammary tumor agent-free animals was approximately 5/4 as compared to a distribution of 15/4 for "typical" tumors. This difference was statistically significant ($P<.05$).

TABLE 2.—*Distribution of "typical" and "atypical" tumors**

Group	Total tumors	Anterior			Posterior			Approximate anterior/posterior ratios	
		Typical	Atypical	No slide available	Typical	Atypical	No slide available	Typical	Atypical
(BALB/cAn × C3H/An)F_1	358	169	26	65	33	25	40	2¾	¼
$C3H_f$/He	243	122	37	11	43	22	8	11.¾	¾
Totals	601	291	63	76	76	47	48	15/4	5/4

*The combined average "atypical" tumor age was 25.7 months. The data were not sufficient to permit an analysis of the effect of age upon the distribution of "atypical" tumors. An analysis of the effect of age upon the distribution of "typical" tumors is presented in the following section of this report.

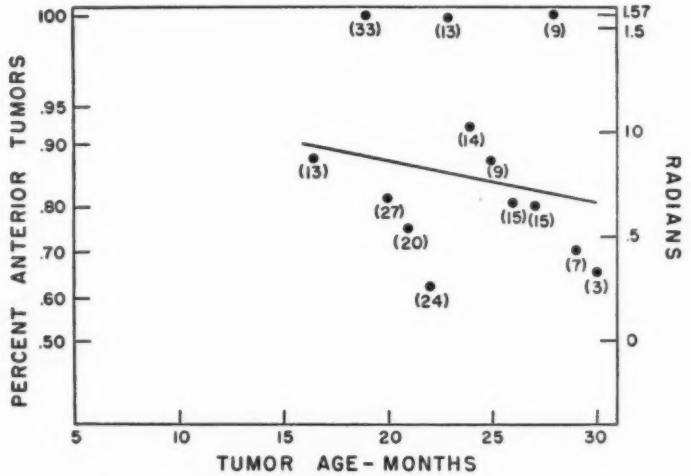
[5] The distribution of "atypical" tumors in the mammary tumor agent-containing strains was not analyzed because these tumors constituted an insignificant percentage of the total tumor population in those strains.

The data were not sufficiently extensive to permit an analysis of the relationship between tumor age and the distribution of atypical tumors. Since the "atypical" tumors seem to have been fairly evenly distributed among the various mammary glands, it must be concluded that the high percentage of anterior tumors found in mammary tumor agent-free mice was not because of, but in spite of the occurrence of "atypical" tumor types.

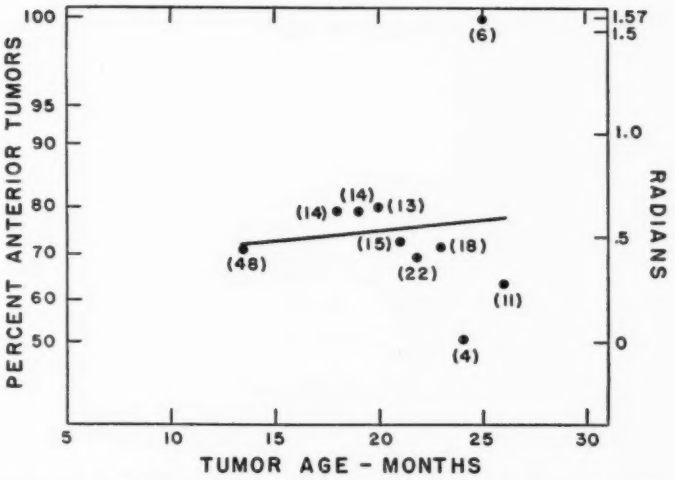
The Relationship Between Tumor Age and Tumor Distribution

The fact that mammary tumor agent-free mice developed tumors at a later average age than did those with the mammary tumor agent gives rise to the possibility that the difference in anterior/posterior tumor ratios might have been a function of the age at which tumors occurred. If the apparent relationship between tumor age and tumor distribution is not fortuitous, it should be possible to demonstrate such a relationship within the internal data from each strain. Under these conditions the other variables such as genetic differences and presence or absence of the mammary tumor agent would be controlled. To this end, the percentage of anterior tumors was plotted against tumor age (in months) for each of the five groups. The arc sine transformation (*7*, *8*) was employed in order to simplify the statistical analysis. Following this transformation, lines of relationship between tumor age and the percentage of anterior tumors were determined for each group. The results are shown in text-figures 1 to 5.

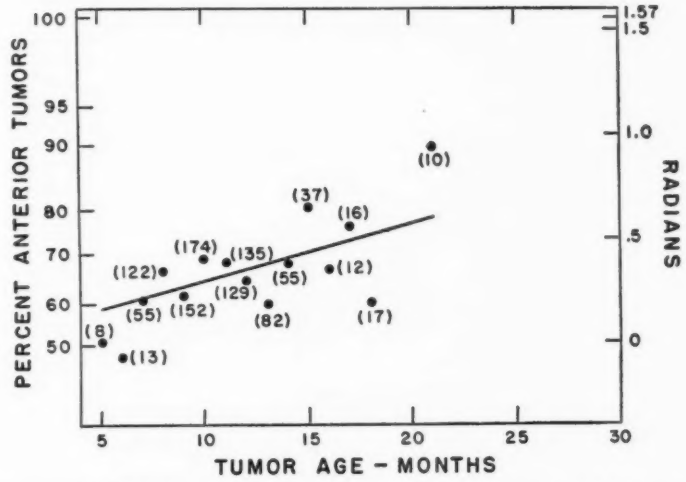
Examination of text-figures 1 to 5 shows that in all three strains containing the mammary tumor agent the relation between the percentage



TEXT-FIGURE 1.—Relationship between percent anterior tumors and tumor age in (BALB/cAn × C3H/An)F_1 hybrids("typical" tumors only). The figure in parentheses indicates the number of tumors upon which the point is based.

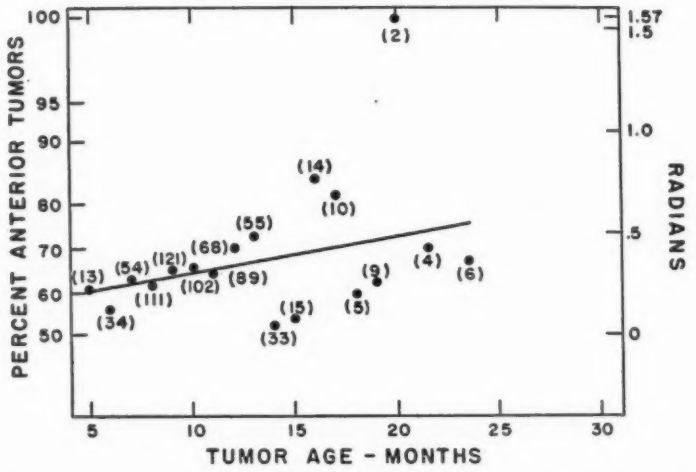


TEXT-FIGURE 2.—Relationship between percent anterior tumors and tumor age in strain $C3H_f$/He ("typical" tumors only). The figure in parentheses indicates the number of tumors upon which the point is based.

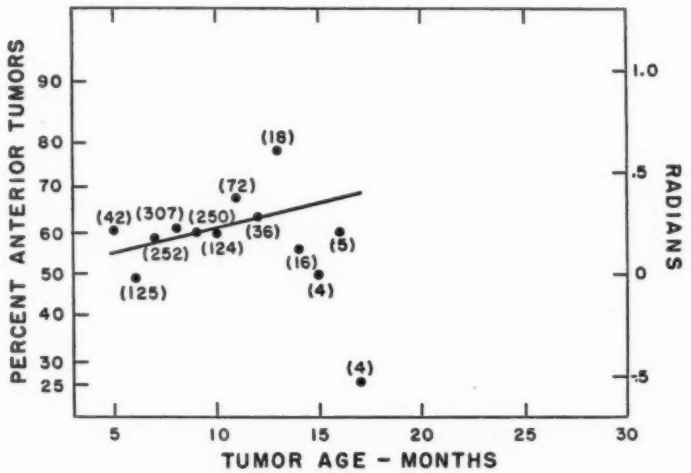


TEXT-FIGURE 3.—Relationship between percent anterior tumors and tumor age in strain A/He. The figure in parentheses indicates the number of tumors upon which the point is based.

of anterior tumors and the tumor age was positive, though significantly different from zero only in the case of strain A/He ($P<.05$). In the case of the two agent-free groups, when "typical" tumors only were considered, one slope was negative and one slope was positive. Neither was significantly different from zero. However, when the slopes of the internal relationships of all five individual groups ("typical" tumors only) were



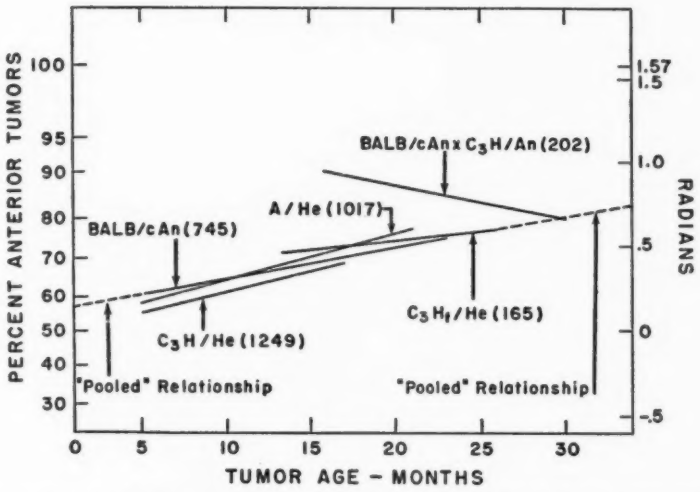
TEXT-FIGURE 4.—Relationship between percent anterior tumors and tumor age in strain BALB/cAn. The figure in parentheses indicates the number of tumors upon which the point is based.



TEXT-FIGURE 5.—Relationship between percent anterior tumors and tumor age in strain C3H/He. The figure in parentheses indicates the number of tumors upon which the point is based.

pooled, the over-all relationship of the percentage of anterior tumors to the tumor age was significantly positive[6] ($P<.05$). None of the individual slopes varied significantly from the pooled over-all slope. This

[6] Average of the weighted individual slopes. Thus, the internal data, on the average, showed a significantly positive slope. Only "typical" tumors were considered because the group differences in tumor distribution have already been shown to be a function of the "typical" rather than of the "atypical" tumors. However, a significantly positive slope would also have been obtained even if the data had included the "atypical" tumors.



TEXT-FIGURE 6.—Resultant relationship when the 5 individual slopes represented in figures 1 to 5 were averaged or pooled. The figure in parentheses indicates the number of tumors upon which the slope is based.

information has been summarized in text-figure 6 which shows: *a*) The slope of the internal relationship within each strain between the percentage of anterior tumors and the tumor age; and *b*) resultant slope of all 5 individual slopes combined or pooled (over-all relationship).

The fact that the over-all relationship (average of the weighted internal relationships) was significantly positive means that a significant portion of the differences among groups in anterior/posterior tumor ratios was accounted for by the tumor age variable. However, analysis also showed that a barely significant difference in anterior/posterior tumor distribution among the groups [due primarily to the (BALB/cAn $\times$ C3H/An)F_1 hybrids] remained after the effect of tumor age had been taken into account. Thus, tumor age was shown to be an important factor determining the percentage of anterior tumors, but this factor was not of sufficient weight to account for all of the differences among the groups. The small remainder (perhaps 15%) must have been due to some other and additional mode of action of the mammary tumor agent or to genetic differences among the groups. A summary table of this analysis of covariance (7) is given below (table 3).

Relationship Between Genotype and Tumor Distribution

In the previous sections it was pointed out that a large portion of the group differences in percentage of anterior tumors was accounted for as a function of varying tumor ages and that the presence or absence of the mammary tumor agent influenced the average tumor age. However, the genotype was also influential. This is demonstrated by the fact that there existed highly significant differences in average tumor ages among all of

TABLE 3.—*Analysis of variance: comparisons among all groups to study the effect of tumor age*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F ratio
Pooled internal regression (within strains, relation between % anterior and age at appearance)	1	1. 785	1. 785	5. 75*
Differences in strain averages, after age effect is taken into account	4	3. 200	. 800	2. 54*
Differences among individual (within strain) slopes	4	1. 262	. 316	Not significant
Deviations (within strains) from individual regressions	59	18. 333	. 311	

*$0.01 < P < .05$

the various strains containing the mammary tumor agent. A statistically significant difference in average tumor ages also occurred between the two mammary tumor agent-free groups. The differences were presumably due to differences in genotype. Thus, both the mammary tumor agent and the genotype influenced the distribution of tumors via an influence on the average tumor age.

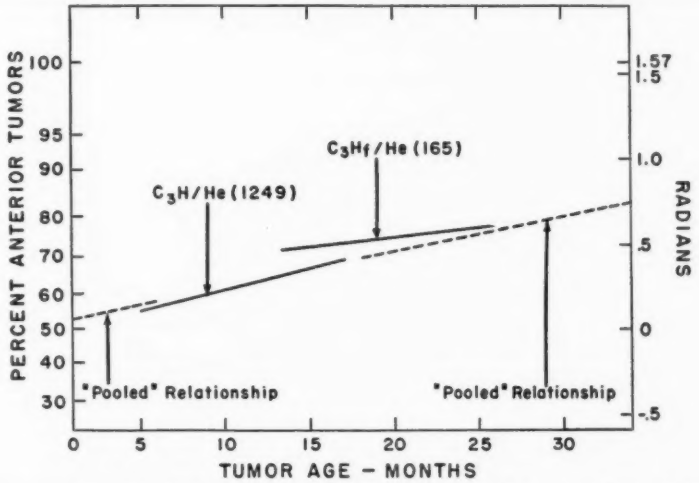
However, as was pointed out in the previous section, the differences in average tumor ages could not account for all of the differences in tumor distribution among the various groups. Either the mammary tumor agent or the genotype (or both) must have influenced the tumor distributions by some mode of action other than by its effect on the average tumor ages. Some notion as to which of these possibilities was more likely was gained by a further analysis of the data for strains C3H/He and $C3H_f$/He.

As has been pointed out, these strains possessed virtually identical genotypes so that differences between them were presumably dependent on the mammary tumor agent. Therefore, if the tumor-age factor could not account for the entire difference in percentage of anterior tumors between these two strains, then the milk agent would have been shown to possess some additional and as yet unidentified mode of action. If, on the other hand, the tumor age satisfactorily accounted for the entire difference between these strains, then it could be generalized that in all cases the mammary tumor agent probably influenced the percentage of anterior tumors *only* via its influence on tumor age. If that were the case, then the small unaccounted-for remainder of difference among all of the groups [*i.e.* between the (BALB/cAn $\times$ $C3H_f$/An)F_1 hybrids and the rest] after tumor age was taken into account would probably have been due to genetic differences, this being the only apparent remaining variable.

Text-figure 7 gives a summary of the analysis of strains C3H/He and $C3H_f$/He ("typical" tumors only). The graph shows the slope of the relationship of the percentage of anterior tumors/tumor age in each strain.

TABLE 4.—*Analysis of variance: comparison of strains C3H/He and C3H$_f$/He to study (by exclusion) the possible effect of genetic differences*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F ratio
Pooled internal regression.....	1	.596	.596	Not significant
Differences in strain averages.....	1	.171	.171	Not significant
Differences among slopes.....	1	.151	.151	Not significant
Deviations from individual regressions.	19	3.8093	.346	



TEXT-FIGURE 7.—Resultant relationship when the individual slopes of strains C3H/He and C3H$_f$/He were averaged or pooled ("typical" tumors only). The figure in parentheses indicates the number of tumors upon which the slope is based.

Also shown is a line representing the "over-all" or pooled relationship [7] for these two strains. Although the average tumor-distribution ratios for each of these two strains were slightly removed from the "over-all" line, the deviations were not statistically significant. It could be concluded that the tumor-age effect accounted for over 95 percent of the difference between the two groups and that the remaining, unaccounted-for difference was not a statistically significant portion. A summary table of this analysis of covariance is given in table 4.

Therefore, since in the case of the C3H/He and C3H$_f$/He strains the tumor-age effect was probably sufficient to account for the differences in tumor-distribution ratios between them, there is no reason to postulate any other and additional mode of action of the mammary tumor agent. It follows logically that since the unaccounted-for differences among the other

[7] Average of the weighted individual slopes.

groups [*i.e.* between (BALB/cAn × C3H$_1$An)F$_1$ hybrids and the rest] were not due to a mode of action of the milk agent other than its effect on tumor age, they were due most probably to a minor influence of differing genotypes. It must be concluded that the genotype influenced the anterior/posterior tumor-distribution ratios in at least two ways: via an influence on the average tumor age and via some other pathway.

Summary and Conclusions

From the statistical analysis of the distribution of 3,612 mammary tumors among the glands of mice from 4 inbred strains and 1 group of F$_1$ hybrids, the following conclusions seem justified:

1) An uneven distribution of mammary tumors (increased anteriorly) among the glands occurred in the absence of the mammary tumor agent, whereas a comparatively even distribution occurred when the agent was present.

2) The degree of unevenness of tumor distribution was largely a function of tumor age. The greater the tumor age, the greater was the percentage of anteriorly occurring tumors. The only influence of the mammary tumor agent on the distribution of tumors was probably by way of its effect on the average tumor ages, which were comparatively low in mammary tumor agent-containing strains. The genotype was also influential in determining the tumor age.

3) "Atypical" tumor types tended to be evenly distributed among the glands in mammary tumor agent-free mice, and their presence could not account for the uneven distribution of mammary tumors.

4) Slight strain differences in tumor distribution unaccounted for by the differences in average tumor ages were presumably dependent upon differences in genotype.

5) The results of this study are consistent with the observations of Pullinger on the distribution of hyperplastic nodules in strain RIII$_b$ mice.

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Mammalian Chromosomes *in Vitro*. IV. Some Human Neoplasms [1,2]

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Mitoses in outgrowing cells from explants *in vitro* show chromosomes in a flat plane which does not require sectioning for ordinary studies. The advantage of using tissue cultures for the enumeration and description of mammalian chromosomes has been enhanced by the technique of treating such material with a hypotonic solution shortly before fixation. This method (*1*) has been used to verify the existence of aneuploidy in the somatic cells of several species of mammals (*2*, *3*).

Since neoplastic tissue is characterized not only by chromosomal anomalies but often also by marked aneuploidy and polyploidy, the newer technique of examining chromosomes in tissue culture appears to offer an important challenge for studies on various types of malignancies. The present work is designed to point out the possibilities of chromosomal studies in highly anaplastic human tissues both as solid tumors and as represented by free cells in ascitic fluid. The purpose of the study was not to provide a comprehensive examination but rather to present a sampling of available material. An abstract of these findings was presented by Hsu and Pomerat (*4*).

Materials and Methods

With the exception of two samples of ascitic fluid which were supplied by the U. S. Public Health Service Hospital (PHSH) in Galveston, all other specimens were received from the John Sealy Hospital (JSH) of Galveston, Texas.

The technique employed for setting up cultures and spreading of chromosomes was essentially the same as that described by Hsu and Pomerat (*1*). Solid tumors were cut into fragments approximately 2 mm. square and mounted on cover glasses (12 × 50 mm.) in plasma clots for culture in roller tubes. Ascitic fluid was centrifuged at 2,000 r.p.m. for

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20 minutes and the sediment from approximately 500 ml. was collected and pooled. One or two large drops of such sediment was incorporated in an embryo extract–plasma clot and roller-tube cultures were made according to the procedure developed by Ivers and Pomerat (*5*) and Pomerat, Nowinski, and Rose (*6*). Cultures of ascitic fluid were allowed to set for about 2 hours to ensure the formation of a firm clot before the liquid nutrient was added.

If a sufficient number of cultures were available, samples were usually fixed on the 2nd to the 6th day of incubation to determine mitotic activity. Such cultures were fixed in methanol and stained with the May-Grünwald mixture followed by Giemsa's stain (*7*, *8*) without hypotonic-solution pretreatment. These sample preparations were of value for observation of mitotic behavior, because mitosis in the treated series was disturbed and the "normal" condition for the tumor mitosis could not be analyzed accurately. In specimens that provided only a limited number of cultures this desirable procedure had to be omitted, and all such cultures were treated with a hypotonic solution and fixed on the 5th to the 9th day of incubation without preliminary check of mitotic activity.

Solid Tumors

I. Sarcoma

(Patient E. A.—JSH No. S-35250, age 44, Negro, male)

This patient had had a recurrent liposarcoma on his left arm that had been surgically treated several times previously. Sections of the present specimen from the same area showed a very pleomorphic tumor but were negative to fat stains.

A photomicrograph of the biopsy section at low magnification is shown in figure 1. Great variation in cell size and tremendous proliferative activity can be readily observed. The mitotic figures are highly abnormal. Figure 2 shows a cell with a multipolar spindle from the same preparation. In none of the mitotic figures were we able to see individual chromosomes, the metaphases and anaphases being represented only by heavily stained blocks of chromatin.

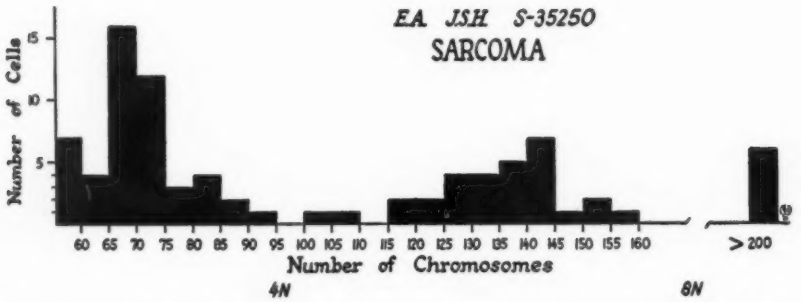
Fourteen roller-tube cultures were prepared on the day the specimen was obtained. One culture tube was fixed with methanol and stained in May-Grünwald–Giemsa on the 3rd and one on the 4th day of incubation without hypotonic-solution pretreatment. On the 6th day of incubation all but one culture tube were treated with a mixture of 20 parts of Gey's balanced salt solution and 80 parts of the same formula without NaCl. The remaining culture was used for studies with phase-contrast, time-lapse cinematography.

Nearly all of the explants produced vigorous outgrowth and abundant mitotic figures. In 6-day cultures mitotic figures were more numerous

than in those incubated for 3 or 4 days. A portion of a culture fixed on the 6th day and treated with the hypotonic solution is shown in figure 3, where three cells of different size are dividing. However, the mitotic figures in cultures washed only in isotonic solution (Gey's) were very similar to those of the original biopsy section. Figure 4 represents one such cell at metaphase. In the cultures treated with hypotonic solution much of the chromosomal detail could be observed, and two such cells have been previously described by Hsu and Pomerat (*1*). Great numbers of polyploid cells were noticed in this tumor (*cf.* fig. 3). Many cells contained short, thin chromosomes as compared with those from cultures of normal tissues similarly treated, and some cells contained chromosome fragments. From cell to cell there was a wide variation in chromosome morphology as well as in number (figs. 5 to 8).

Levan and Hauschka (*9*) believe that in their mouse ascites tumors many cells undergo endomitosis. Superficially, the dividing cells in preparations treated with our hypotonic solution resemble those of Levan and Hauschka's endometaphase. While undoubtedly endomitosis occurs in tumors as well as in normal tissues, the cells in our slides of this sarcoma do not represent endomitosis because in the cultures washed with isotonic solution all mitotic figures showed evidence of spindles (*cf.* fig. 4). A cinematographic sequence of this tumor also showed anaphasic movement, although the cleavage occurred prematurely; *i.e.*, cytokinesis began at very early anaphase. On the other hand, in the preparations that received the hypotonic-solution treatment no such equatorial arrangement of chromosomes was observed. It is therefore concluded that the untreated cells illustrated in figure 4 and the treated cells from the same tumor shown in figure 3 both represent but one type of living cell. Hence, the appearance of the underdeveloped chromosomes in the treated series demonstrates a characteristic feature of spiralization and condensation of the chromosomes rather than endomitosis.

Chromosome counts were not easy to perform in random samples because a number of cells containing fragments and underdeveloped chromosomes were not suitable for reliable counts. Cells displaying well defined chromosomes were counted. The error for numbers below 100 is not more than ± 2 but in the higher ranges it is expected to be considerably greater. Among 84 cells counted not a single cell contained the diploid number of chromosomes of man, 48. The lowest number counted was 57 and the highest 346. Actually, many cells had numbers of chromosomes far in excess of this limit (*e.g.*, the largest cell in figure 3 whose number probably was of the order of 1,000), but in most such cells the chromosomes were so numerous that counts were not practicable. Therefore, the histogram (text-fig. 1) tends to show more low values than high ones. However, even with the 84 cells counted, a wide range in chromosome number was demonstrated. There was a rather low peak near 70 and a lower one at approximately 140, but not a single cell was found that contained the tetraploid number, 96, and even the range from 90 to 100 was represented by only two cells.



TEXT-FIGURE 1.—Distribution of chromosome numbers of sarcoma (Case I) cells in tissue culture.

II. Squamous-Cell Carcinoma of the Submaxillary Gland (Poorly Differentiated)

(Patient E. B. G.—JSH No. 3403-I, age 59, white, male)

The patient had had radiation therapy for his intra-oral lesion and had symptoms of postradiation necrosis of the mandible. The tissue sample was set up in roller-tube cultures in the usual way. Due to the high proteolytic activity of the epithelial cells many explants fell off the cover-slips, but a few survived to give rise to sheets of epithelium. Figure 9 shows a portion of the outgrowth of a culture fixed on the 6th day of incubation which had had pretreatment with a hypotonic solution. The highly lobated nucleus in the cell at the lower portion of figure 9 is rather exceptional, because the nuclei of most of the epithelial cells tend to be round or oval. They usually appeared slightly granular in texture, containing one or two nucleoli, rarely three or more. Multinucleation was quite common, indicating a high frequency of failure in cytokinesis.

Judging from the sizes of the nuclei there was definite evidence of polyploidy in these tumor cells. However, chromosome counts were impossible because all the cells in mitosis showed minute fragments as represented in figures 10 and 11. Even during prophase (fig. 12) this phenomenon can be clearly seen. Marked fragmented chromosomes were found to be characteristic in more than 200 divisions that were examined.

III. Bronchogenic Carcinoma of Left Lung

(Patient C. G. L.—JSH No. 19342-I, age 57, white, male)

The patholigist's report revealed a poorly differentiated squamous-cell carcinoma of the bronchial wall. There were round cells with vesicular nuclei containing prominent nucleoli. Cytoplasm was moderately abundant and the cells tended to occur in nests which did not show keratohyalin.

Like the preceding specimen, most of the explants in the roller-tube cultures fell off the cover-slips quickly and there were few surviving cultures. The epithelial cells fixed after treatment with the hypotonic solution on the 5th day of incubation showed variation in size and coarse texture of nuclei and cytoplasm. In many cultures the cells in the out-

growth zone did not form epithelial sheets. However, in a few cultures where sheets were present (fig. 13) "nest" or "pearl" formation was frequently observed. Vacuolation, multinucleation, and lobation of nuclei were very common.

Mitotic figures were not abundant in the 5-day cultures. The chromosomes appeared short, malformed, and ragged in outline, though not in the form of minute fragments (fig. 14). No critical counts were made for this tumor.

IV. Granulosa-Cell Carcinoma of the Right Ovary

(Patient A. R.—JSH No. 9520-I, age 51, Negro, female)

Only 5 roller-tube cultures were available for this tumor but nearly all explants produced good outgrowth. The cultures were fixed on the 9th day of incubation, with hypotonic-solution pretreatment. The epithelial cells formed compact clusters and sheets, and showed dense staining reaction. Often they formed large sheets surrounded by spindle-shaped, rather faintly staining fibroblasts (fig. 15). Occasionally, small islands or even single cells of tumor elements could be found.

Mitotic figures were observed in a few cells and their chromosomes appeared similar to those of normal tissues under the same treatment. Figure 16 represents a polyploid cell in mitosis. No counts were made for this tumor because the cells were too few to be representative of the population.

V. Bilateral Papillary Serous Cystadenocarcinoma of the Ovaries

(Patient P. N.—JSH No. 8564-I, age 49, white, female)

Three cultures were sampled on the 6th day of incubation without the hypotonic-solution pretreatment, and the rest of the cultures were treated and fixed for chromosome study on the 8th day.

Epithelial sheets of various sizes ranged from small patches of only a few cells to extremely large masses. Unlike other carcinomatous cells in culture, which are usually clumped together with restricted cytoplasmic areas, the cells of this tumor spread out thinly with an enormous extension of cytoplasm. Figure 17 shows a rather densely packed area from a 6-day culture fixed with methanol and stained with May-Grünwald–Giemsa. The cytoplasm appeared rather homogeneous in texture, rarely containing vacuoles; the nuclei generally appeared roundish or oval, although some were irregularly outlined or even lobated. Variation in nuclear size, common in malignancies, was also observed. However, this tumor exhibited a peculiar nuclear phenomenon which was not as common in any other specimen in the series; namely, the formation of halo-like clear areas around the nucleolar bodies. Figures 18 and 19 illustrate such nuclei (fixed on the 8th day with hypotonic-solution pretreatment) at high magnification. There seemed to be two varieties of such bodies, one usually large and heavily stained by hematoxylin and the other lightly stained with approximately the same degree of chromaticity as

the remaining nucleus. The halos were especially marked around the lightly stained bodies. In figure 18 the two lightly stained masses appear roundish, structureless, and contain a ball-like vacuole homogeneous in structure. In figure 19, however, the lightly stained bodies show coils resembling chromosomal material. The heavily stained structures also had hyaline areas but these were relatively smaller and less homogeneous. Slide preparations stained with May-Grünwald–Giemsa without hypotonic-solution pretreatment also showed this phenomenon (*cf.* fig. 17). According to Jacobson and Webb (*7, 8*) this stain can serve to determine the two types of nucleoproteins—staining reddish for desoxypentosenucleoprotein and deep blue for pentosenucleoprotein. The heavily stained bodies were deep blue, hence probably consisted mainly of pentosenucleoprotein of nucleolar material; and the lightly stained bodies were reddish, hence of desoxypentosenucleoprotein or chromatin substance.

Only a few dividing cells were present (fig. 20) and the chromosomes in these appeared to be similar to those in cells dividing in normal tissue. No counts were made.

Cells in Ascitic Fluid

In experimental animals it has been demonstrated by many authors, notably Hauschka and Levan, Goldie, Klein, Makino, and their associates, that ascites tumors provide excellent material for various studies on neoplasms. As stated by Klein (*10*), in the peritoneal fluid of the ascites tumors of the mouse the percentage of the tumor cells increases after a few fluid transplant generations and remains at a particular level. This was especially well demonstrated in the Krebs 2 carcinoma. Nontumor elements such as various blood and mesothelial cells are always present in the fluid.

In man, ascitic fluid can often be obtained from patients with a wide range of pathologic conditions offering valuable material for cytologic studies (*6*). In tissue culture medium the mesothelial cells soon transform into spindle-shaped fibroblasts, but the tumor cells retain their original characteristics and usually can be recognized easily both in the living state and in stained preparations.

VI. Adenocarcinoma of the Stomach

(Patient G. McC.—PHSH No. 41594, age 55, white, male)

This fluid specimen was received before the technique of pretreatment with hypotonic solution had been developed and the cultures, sampled every two or three days, were all fixed in methanol and stained with May-Grünwald–Giemsa.

The tumor cells, in clusters of varying size in the original fluid specimen, formed flattened epithelial sheets by the 2nd day of incubation (fig. 21). The malignant cells could be identified readily by their coarse and densely staining cytoplasm, rare processes, high incidence of multinucleation, irregularly outlined nuclei of various sizes and of rough texture [similar to the type A cells of Barigozzi and Dellepiane (*11*)], and by marked

mitotic activity. This phenomenon obtained *in vitro* for malignant cells is rather significant when compared with nonmalignant epithelial cells. A number of them, presumably of glandular origin, formed rather compact papilla-like cell clusters which were extremely densely stained (fig. 22). Figure 23 shows a cell at high magnification with a micronucleus, irregular nuclear outline, and numerous vacuoles. The morphology of the tumor cells changed very little after they had flattened into sheets. Some of the clusters maintained their compactness even after 15 to 20 days of incubation.

Marked mitotic activity was recorded in the 2-day-old cultures. In fact, in this particular tumor the frequency of division of the malignant cells was much higher in young cultures and the cells ceased to proliferate after about 10 days of incubation (table 1). By that time the fibroblasts had developed a tremendous rate of outgrowth and the tumor cells were almost surrounded. This is analogous to the observation reported by Barnes and Furth (*12*) for a mouse leukemia.

TABLE 1.—*Percentage of epithelial cells in mitosis, each sample being based upon more than 1,000 cells*

Days of culture	2	4	6	9	12	15	20
	2.9	1.9	1.3	0.4	0.0	0.0	0.0

Since the preparations were not pretreated with hypotonic solution, no chromosomal detail could be observed and enumeration of chromosomes was not possible. However, the bulk of the metaphase plates, both in side and polar views, strongly indicated a great variation of chromosome number among the cells. Many were obviously polyploid. A rough method of estimation was made by recording arbitrarily the nuclear size in several classes. Disregarding the micronuclei, Class I nuclei were those of regular size, presumably diploids or aneuploids of the diploid rank. Class II nuclei were tetraploids or elements close to tetraploids, having a nuclear volume approximately twice as great as that of members of Class I. Class III nuclei were octoploids or the like, and Class IV 16-ploids. This estimate, though only an approximation, was employed to show the trend of polyploid formation in the cultures. From table 2 it appears that the percentage value of polyploids in the cell populations fluctuated slightly without any definite tendency to increase or decrease during the incubation period.

TABLE 2.—*Percentage of nuclei of different size classes in tumor cells* in vitro

Days of culture	2	4	6	9	12	15	20
Class I	96.0	92.0	93.1	95.2	94.1	95.6	95.3
Class II	3.7	6.8	6.0	4.5	5.4	4.0	3.9
Class III	0.3	0.9	0.8	0.3	0.4	0.3	0.7
Class IV	0.0	0.3	0.1	0.0	0.1	0.1	0.1
Number of cells counted	1,872	1,813	1,444	1,258	1,472	1,323	1,172

In his studies on the mitotic behavior of cancer cells, Koller (*13*) described several categories of anomalies. Examples of practically all of these were found in stained cultures of this patient's tumor. However, complete suppression of the spindle was not common. Only one metaphase was observed to be of the c-mitosis type in more than 200 cells which were examined. Multipolar spindles were very common (fig. 24). A great number of metaphases and anaphases showed one or more laggards, and micronuclei were consequently extremely abundant. Nondisjunction was to be expected but it was difficult to make direct observations except in cases where unequal division was very apparent. Only a very small fraction of the cells showed normal condensation and nucleic acid charge. Practically all showed some degree of over-spiralization and over-condensation. Many chromosomes appeared spherical and heavily charged with nucleic acid. Among the multinucleated cells that could be interpreted as the result of abnormal cell cleavage, those with two nuclei were most common. About 5 percent of the cells were binucleate or multinucleate and, like the polyploid cells, no definite increase or decrease of the proportion of these cells was noticed in the total cell population.

VII. Adenocarcinoma of the Ovary

(Patient E. C.—JSH No. 10168, age 62, white, female)

Most of the tumor cells were in small clusters and appeared to degenerate during the first few days of culture. There was marked liquefaction of the clot around the clusters. Between the 7th and the 10th day of incubation the surviving carcinoma cells proliferated actively and many mitotic figures were observed. Figure 25 shows a small cluster of 8-day-old tumor cells *in vitro* with 8 dividing cells, one of which is enlarged in figure 26.

The chromosomes appear deformed in most cases (figs. 26 and 27) and their enumeration was not feasible. In extreme cases the chromosomes were broken up into minute particles as in some of the other tumors described. In figure 28 the dividing cell shows numerous particles and a spindle is still present.

VIII. Metastatic Melanoma (Probably Achromatic Type)

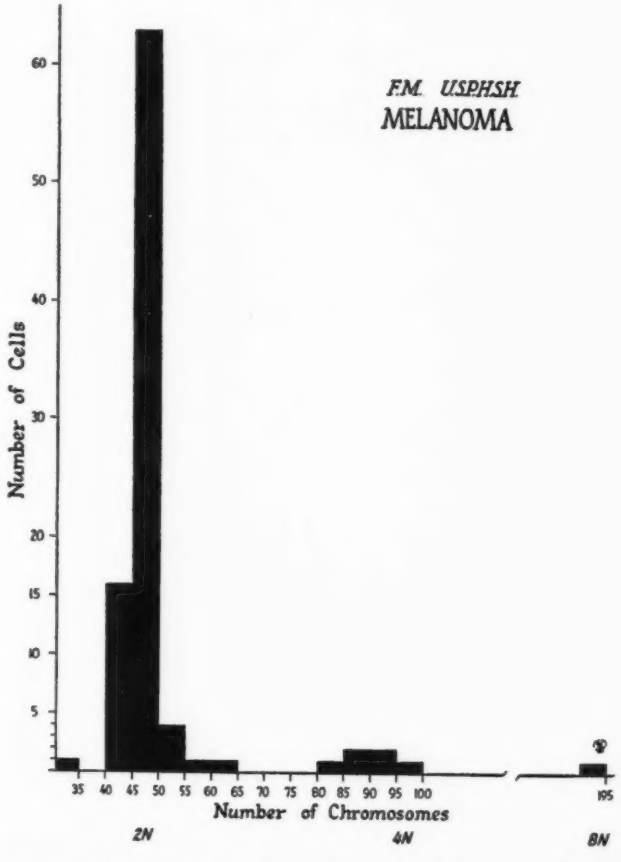
(Patient F. M.—PHSH No. 45894, age 54, white, male)

Malignant cells were not extremely abundant in the original fluid and appeared solitarily or in very small clusters. Figure 29 represents a group of tumor cells found in the culture after 4 days of incubation together with a number of wandering elements and the cytoplasmic process of a spindle cell. These tumor cells appeared relatively rather more uniform than the other neoplasms in this series, being generally roundish and rarely possessing processes or vacuoles. The nuclei were also quite uniform in size, with occasional apparent polyploids.

Preparations stained after treatment with the hypotonic solution (10% Gey's solution in 90% Gey's solution without NaCl was used for this tumor) showed that the shape, length, and nucleic acid charge of the

chromosomes in most of the cells resembled those from normal epithelial cells *in vitro*. Figure 30 represents a well-spread metaphase plate. Counting was not difficult in this type of cell. Some individual cells, however, showed some distortion of chromosome morphology (fig. 31). Figure 32 illustrates a polyploid cell in division.

The chromosome numbers for 92 cells are given in text-figure 2, with a counting error of not more than ± 2. In contrast to the results obtained for the sarcoma (Case I), in which none of the cells had 48 chromosomes or even approximately this number, 34 cells of 92 (approximately 37%) had the diploid number. Furthermore, the variation of the chromosome number was not wide, since most of the cells fell within the range of aneuploidy reported for normal tissues (*14*, *3*). A few cells, however, were somewhat removed from the normal range of variation. One cell was found to have 35 chromosomes and another contained 61. As a



TEXT-FIGURE 2.—Distribution of chromosome numbers of melanoma (Case VIII) cells in tissue culture.

whole, however, the distribution curve followed rather closely the normal pattern of chromosome numbers, $2n$, $4n$, $8n$, etc.

Discussion

It has long been known that malignant cells retain their characteristics in tissue culture medium (*15*). The pattern of growth may be modified (*16*) but the malignancy and the basic morphologic and physiologic properties of cancer cells probably are not changed as a result of short-term residence *in vitro*, and much information gathered from the study of cultures may be applied to the *in vivo* situation. However, this problem deserves further study with tissue culture techniques.

It has been pointed out (*17–19*) that exfoliative cytology can be advanced a step beyond the usual static phase to a dynamic stage by means of tissue culture methods. This permits extended examination of chromosome complements, and in addition the morphology of the dead, fixed cells, their staining reactions, and other features can be examined. Their activities and reactions to various environmental factors can be recorded by means of fairly simple techniques. Not only the detailed cell structure of tissue exudates, such as nipple secretion or the cells in body fluid, but elements in solid tumors may be more advantageously examined *in vitro* than with sectioned biopsies alone.

The present article illustrates what can be observed with regard to the nuclear phenomena of tumor tissues in culture. Mitotic abnormalities can be studied without the risk of damage by the microtome knife in sectioning or by the crushing that sometimes occurs in conventional squash preparations. Cinematographic records may be of special value for the analysis of chromosomal movements. After the addition of a hypotonic solution, mitotic behavior is obscured by the spreading of the chromosomes, but the chromosomes in many cells can be counted and nuclear phenomena that otherwise are not clearly visible can be revealed. This is demonstrated, for example, with regard to the reported stickiness of the chromosomes in cancer cells. Without prefixation treatment with a hypotonic solution, we also observed this stickiness in the cells *in vitro*, but after spreading the chromosomes an entirely different picture was obtained. The aggregated chromosomes proved to be discrete minute particles crowded together (*cf.* figs. 3 and 4). The occurrence of cells with such chromosome fragments in tumors is not a new finding. Darlington and La Cour (*20*) successfully photographed a cell from a human stomach tumor in which all the chromosomes were "centric fragments." However, the wide occurrence of such fragmentation in our material was quite unexpected. In Case II, who had received radiation and showed necrosis, the fragmentation might be explained as the result of heavy radiation damage which broke the chromosomes, but many cells of Case I, who had never received any radiation treatment, also showed such fragmentation. A normal cell, after receiving heavy doses of radiation and losing all of the chromosome material save the centromeric pieces, presumably would not be capable of surviving and reproducing. Other-

wise, it would mean that centric fragments are all that is needed for cell maintenance and reproduction. However, in Case II all the cells that were dividing showed only the fragmentation type of chromosome which should not now be considered merely as due to chance nor as a pure radiation effect. It seems that this extreme fragmentation may have been characteristic of this particular tumor.

It is interesting to note that each tumor seemed to have its characteristic features in terms of chromosome morphology. In addition to the peculiarities described for Case II, Cases I and VII were very similar in having underdeveloped and, in terms of staining ability, rather undercharged chromosomes in the majority of cases and, only occasionally, completely fragmented chromosomes. Case III had malformed, short chromosomes and Cases IV, V, and VIII showed normal chromosomes.

Levan and Hauschka (*21*) and Hauschka and Levan (*22*) found that in the ascites tumors of the mouse the cells retained their characteristic pattern of polyploidy and aneuploidy throughout many transplantation generations. The DBA lymphoma and 6C3HED lymphosarcoma had their chromosome numbers near the diploid region, whereas in the Ehrlich carcinoma and many other tumors the majority of the chromosome counts fell in the tetraploid range. In the Krebs 2 carcinoma the distribution was found to be very much dispersed. Similar conclusions have been reached by Makino (*23*) and Makino and Kanô (*24*) on rat tumors for both chromosome numbers and chromosome morphology, leading these Japanese authors to suggest the term "stem-line" for the series of cells responsible for the specific chromosome pattern. In our collection of human tumors *in vitro* the cells also exhibited rather definite patterns of chromosome number for the particular tumor. Besides the morphologic abnormalities of the chromosomes, the sarcoma of Case I was characterized by a wide range of chromosome numbers, from hyperdiploids to extremely high polyploids or heteroploids without a prominent peak or having multiple peaks in the distribution curve. Perhaps a similar condition existed in Case VII though no actual data were available. In contrast, the distribution of the chromosome numbers in Case VIII was concentrated at the diploid range which is analogous to the DBA lymphoma of the mouse. In fact, the distribution curve of Case VIII did not deviate far from that expected for normal tissues.

Summary

The treatment of cells in tissue cultures of human neoplasms with a hypotonic solution prior to fixation spreads the chromosomes and thereby enhances the opportunity to study them and to make counts. Results from five solid tumors and three ascitic fluid specimens are given.

Chromosomes appeared similar to those in healthy tissues in cultures of a granulosa-cell carcinoma of the ovary, a papillary cystadenocarcinoma of the ovary, and a metastatic melanoma. Chromosomes were ragged and short in a bronchogenic carcinoma of the squamous type. In a sarcoma and an adenocarcinoma of the ovary, the chromosomes were

deformed with great numbers of fragmentary particles in many of the cells. In a squamous-cell carcinoma of the submaxillary gland nearly all the cells showed extensive fragmentation.

The distribution of chromosome numbers in the sarcoma showed low modes near the values of 70 and 140, and no cell (out of 84) could be found with the diploid or tetraploid number. On the other hand, the chromosome counts of a melanoma demonstrated a distribution close to that found for normal tissues.

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PLATE 69

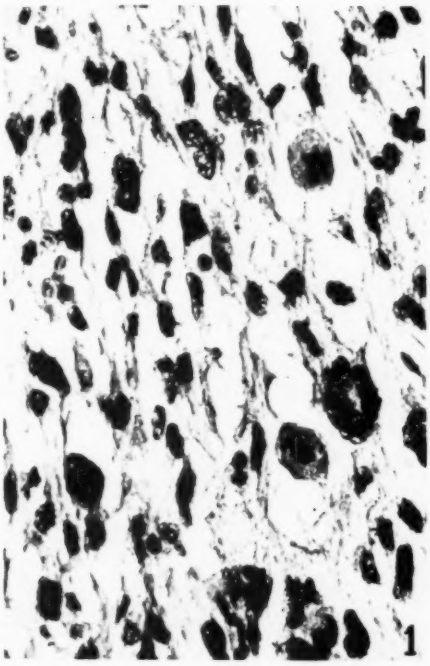
Sarcoma (*Patient E. A.*)

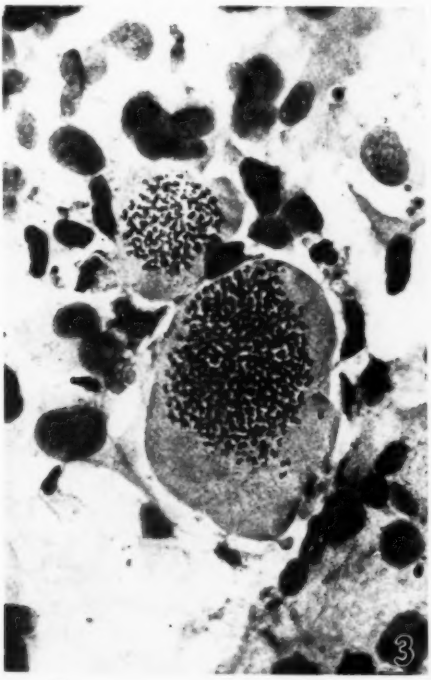
FIGURE 1.—A section of the biopsy, showing nuclear structure, variation in cell and nuclear size, and mitotic activity. × 800

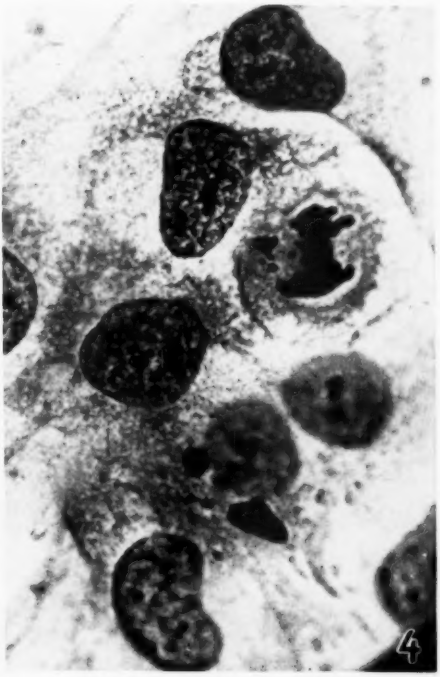
FIGURE 2.—A cell at metaphase from a section of the biopsy showing a multipolar spindle and "stickiness" of chromosomes. × 3,650

FIGURE 3.—A portion of a zone of outgrowth, 6-day-old culture treated with a hypotonic solution before fixation. Note the great variation of chromosome numbers in the three dividing cells. Helly-Zenker fixation, Delafield hematoxylin. × 800

FIGURE 4.—A portion of a zone of outgrowth, 3-day-old culture treated with an isotonic solution before fixation. The cell in metaphase exhibits features similar to the cell shown in figure 2. Methanol fixation, May-Grünwald–Giemsa. ×2,190







Hsu

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PLATE 70

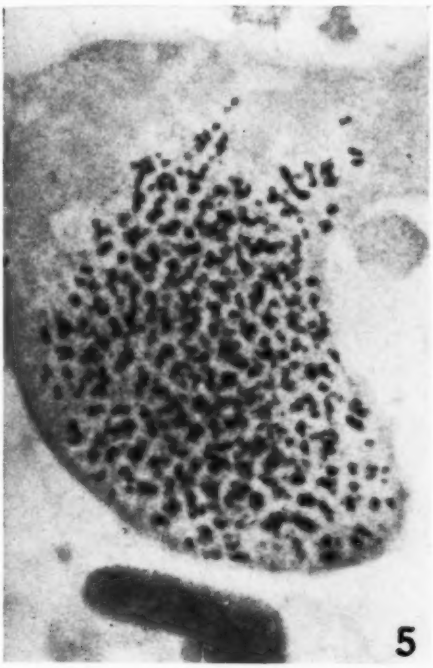
Sarcoma (Patient E. A.), 6-day-old cultures treated with a hypotonic soution before Helly-Zenker fixation and stained with Delafield hematoxylin. × 2,190

FIGURE 5.—A high-polyploid cell with deformed and undercondensed chromosomes.

FIGURE 6.—An aneuploid cell (67 chromosomes) showing chromosomes of relatively normal appearance.

FIGURE 7.—Two low-grade polyploid cells. Note the spiral structure of some chromosomes.

FIGURE 8.—A high-polyploid cell with deformed and undercondensed chromosomes. The chromosomes in the right area are in the form of tiny particles and probably belong to an additional nucleus.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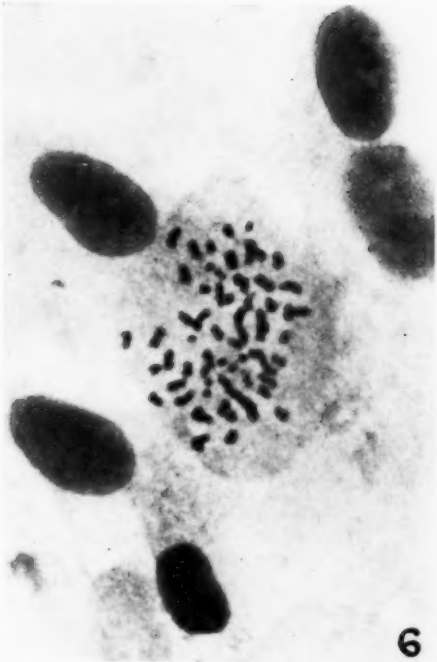
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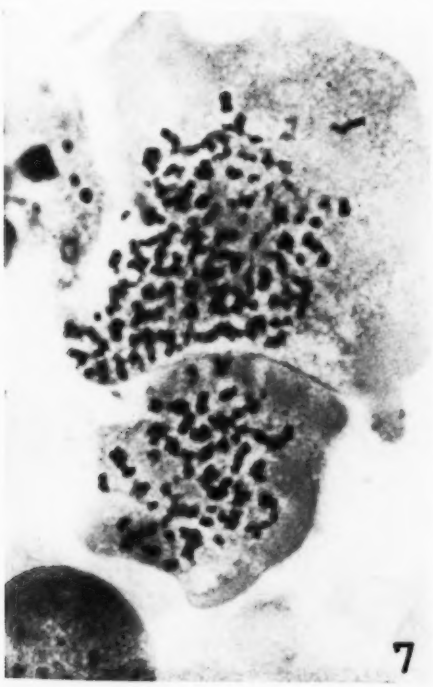
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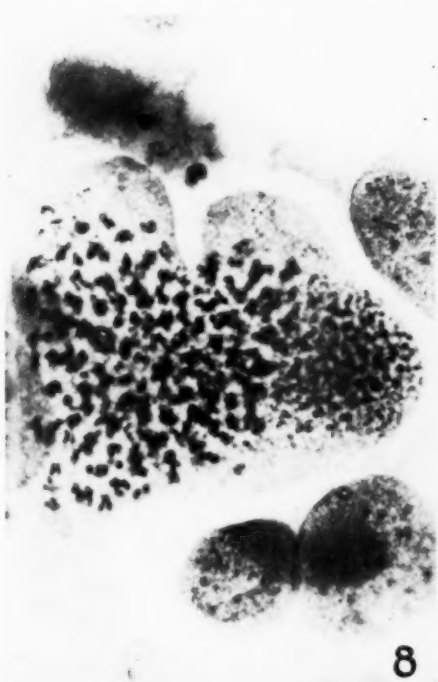
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PLATE 71

Squamous-cell carcinoma of the submaxillary gland (Patient E. B. G.), 6-day-old cultures treated with a hypotonic solution before Helly-Zenker fixation and stained with Delafield hematoxylin.

FIGURE 9.—A portion of a zone of outgrowth. × 800

FIGURE 10.—The mitosis in figure 9 at higher power and in another axis. Note the minute particles of the chromosome material. × 3,650

FIGURE 11.—Another cell, showing chromosome morphology similar to that in figure 10. × 3,650

FIGURE 12.—Another cell, prophase. × 3,650

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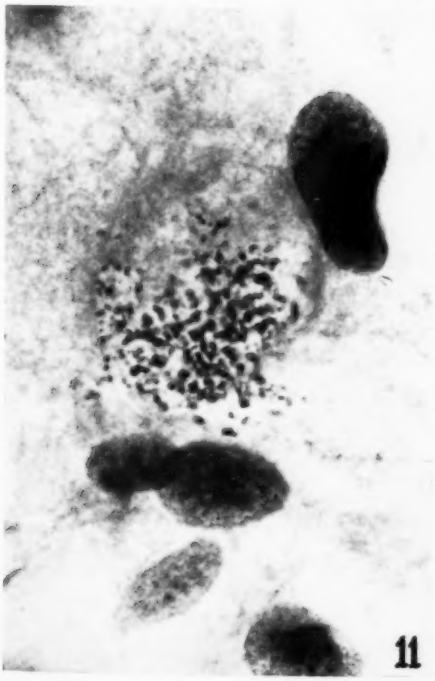
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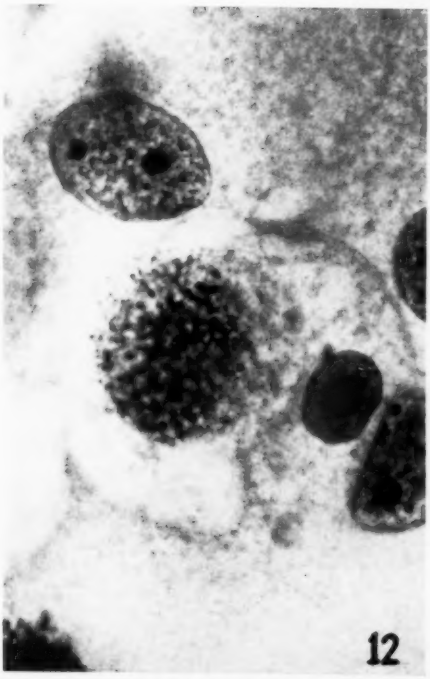
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PLATE 72

Squamous-cell carcinoma of lung (Patient C. G. L.), 5-day-old cultures treated with a hypotonic solution before Helly-Zenker fixation and stained with Delafield hematoxylin.

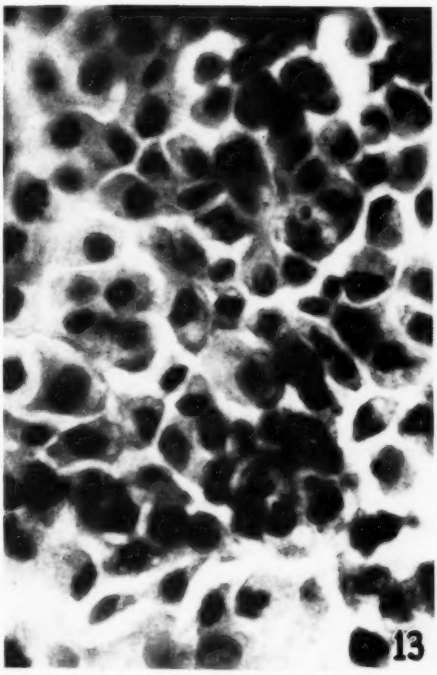
FIGURE 13.—A portion of a zone of outgrowth. × 800

FIGURE 14.—A cell in mitosis with deformed, ragged chromosomes. × 3,650

Granulosa-cell carcinoma of ovary (Patient A. R.), 9-day-old cultures treated with a hypotonic solution before Helly-Zenker fixation and stained with Delafield hematoxylin.

FIGURE 15.—A portion of a zone of outgrowth. × 800

FIGURE 16.—A polyploid cell in mitosis. × 3,650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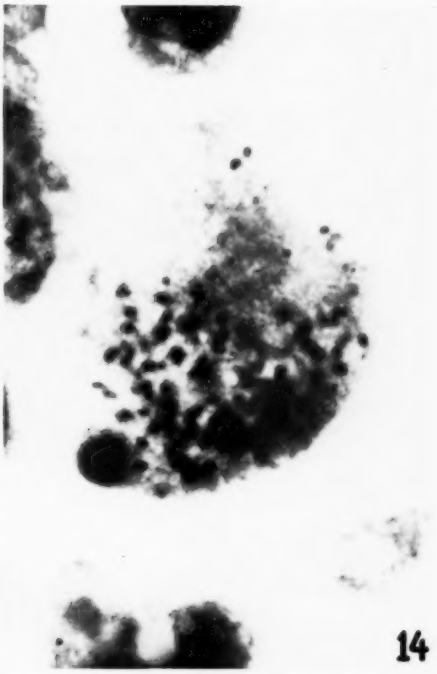
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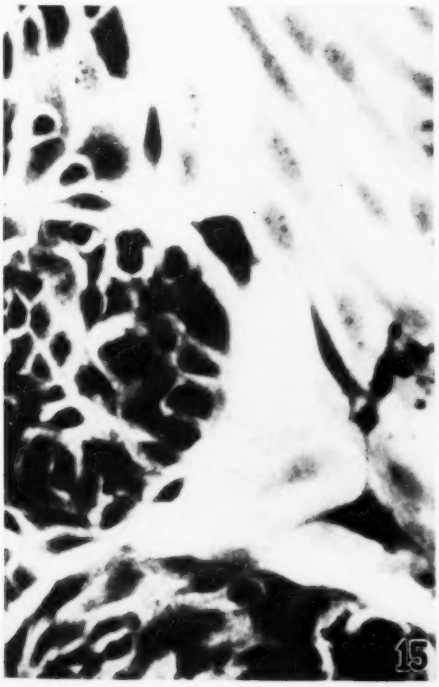
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PLATE 73

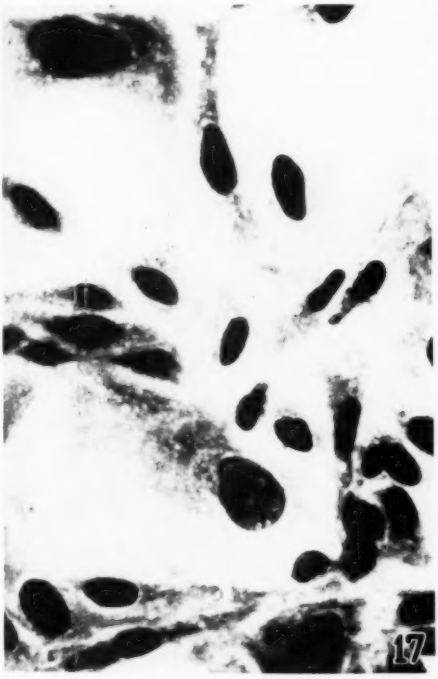
Papillary serous cystadenocarcinoma of ovary (Patient P. N.)

FIGURE 17.—A portion of a zone of outgrowth, 6-day-old culture treated with an isotonic solution before methanol fixation. Note halos around the nucleolar bodies. May-Grünwald–Giemsa. × 800

FIGURE 18.—Nuclei at high magnification, 8-day-old cultures treated with a hypotonic solution before Helly-Zenker fixation. Delafield hematoxylin. × 3,650

FIGURE 19.—Same as figure 18.

FIGURE 20.—A cell in mitosis, 8-day-old culture treated with a hypotonic solution before Helly-Zenker fixation. Delafield hematoxylin. × 3,650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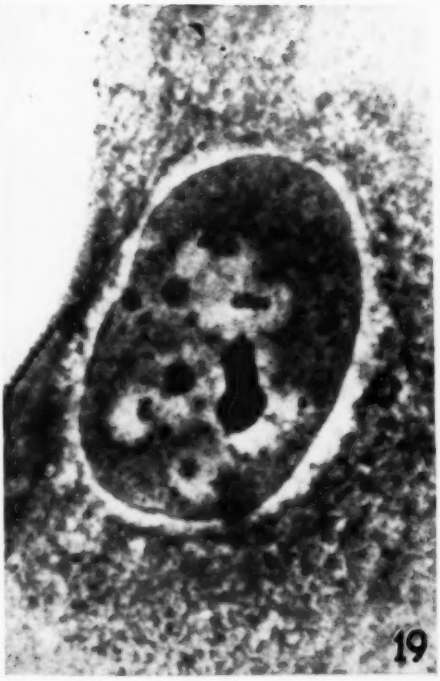
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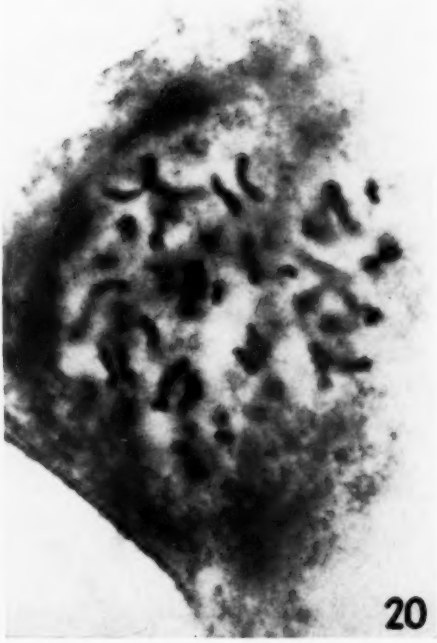
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PLATE 74

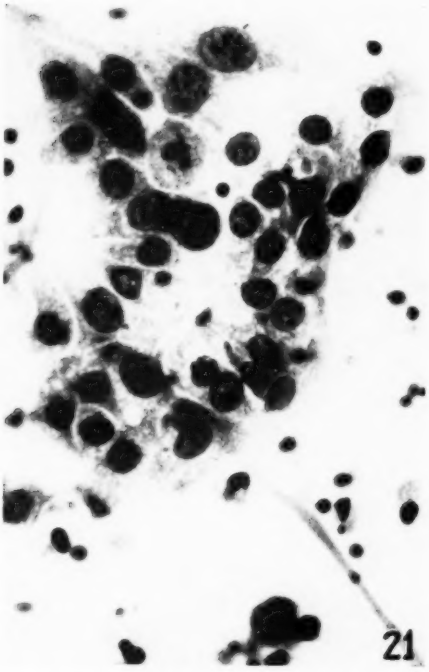
Ascitic fluid from adenocarcinoma of stomach (Patient G. McC.), 2-day-old cultures treated with an isotonic solution before methanol fixation and stained with May-Grünwald-Giemsa.

FIGURE 21.—Clusters of tumor cells and nonmalignant elements. × 800

FIGURE 22.—Same as figure 21.

FIGURE 23.—A cell at high magnification. Note the large vacuoles and the presence of a micronucleus. × 3,650

FIGURE 24.—A tripolar anaphase. ×3,650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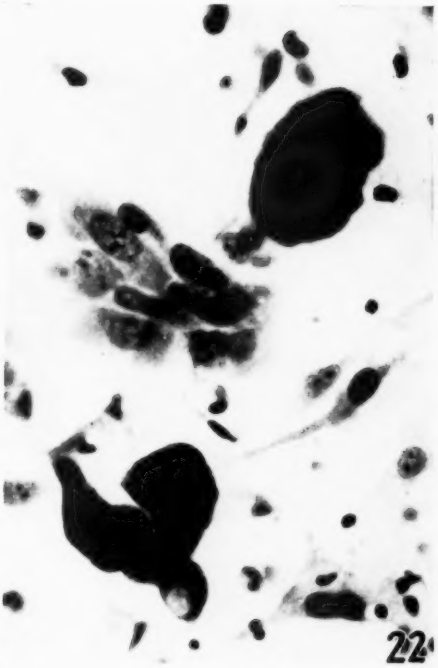
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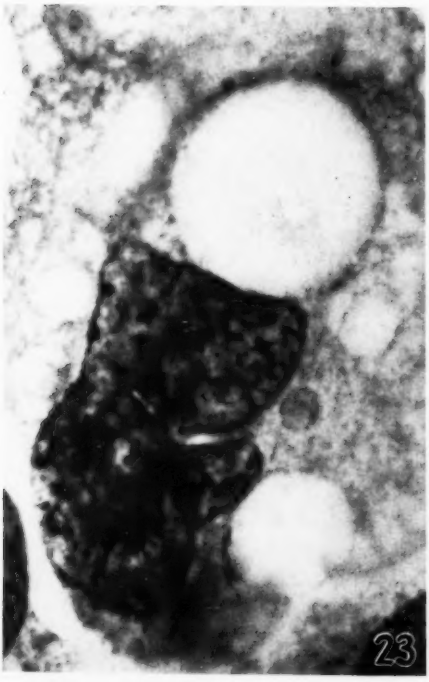
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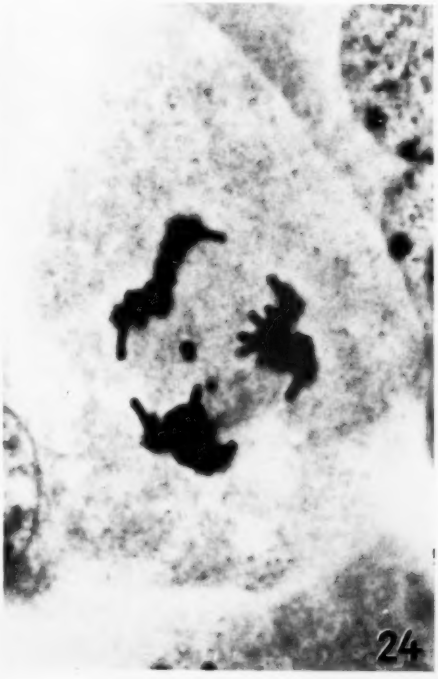
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PLATE 75

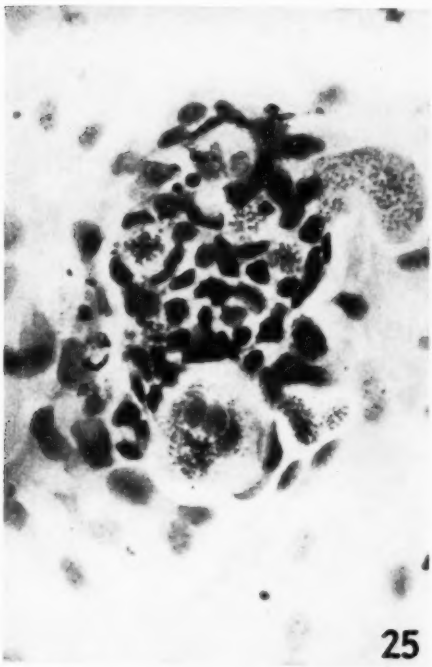
Ascitic fluid from an adenocarcinoma of ovary (Patient E. C.), 8-day-old cultures treated with a hypotonic solution before Helly-Zenker fixation and stained with Delafield hematoxylin.

FIGURE 25.—A cluster of malignant cells. Note numerous mitoses. × 800

FIGURE 26.—A cell from figure 25 at higher magnification. × 3,650

FIGURE 27.—An early anaphase with undercharged chromosomes. × 3,650

FIGURE 28.—Metaphase, showing minute fragments and the spindle. × 3,650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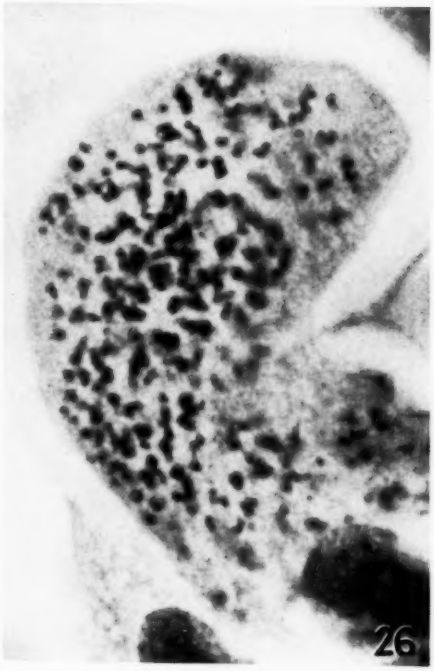
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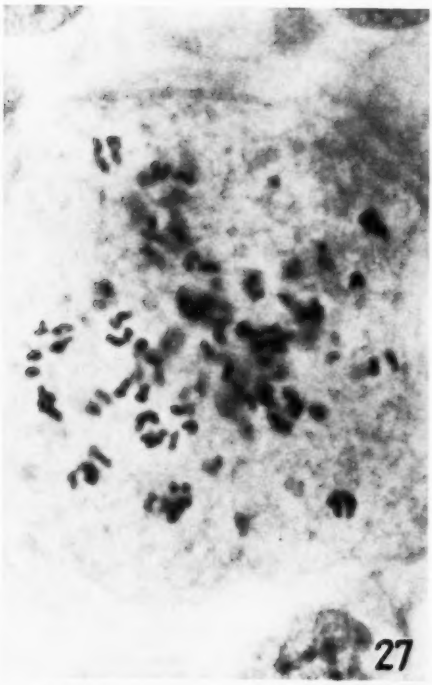
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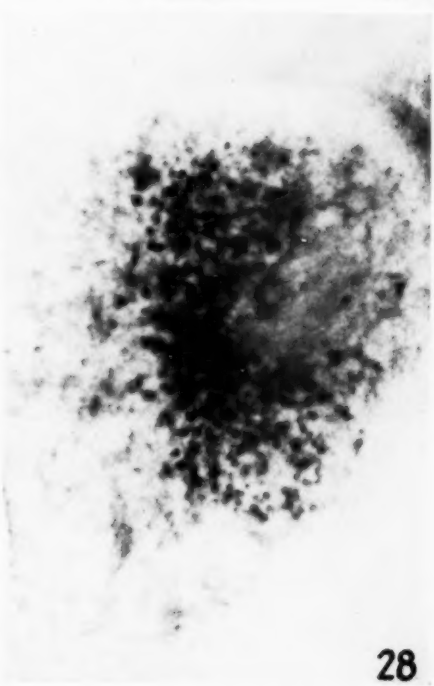
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PLATE 76

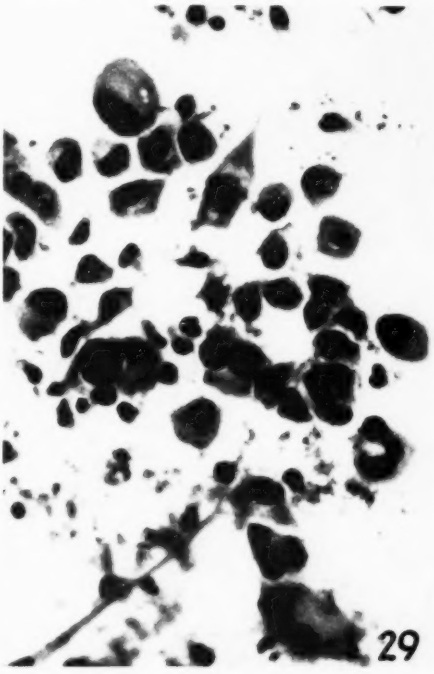
Ascitic fluid from a metastatic melanoma (Patient F. M.)

FIGURE 29.—A group of tumor cells and nonmalignant elements, 4-day-old culture treated with an isotonic solution before methanol fixation. May-Grünwald–Giemsa. × 800

FIGURE 30.—Metaphase, apparently normal, 8-day-old culture treated with a hypotonic solution before methanol fixation. May-Grünwald–Giemsa. × 3,650

FIGURE 31.—Abnormal mitosis, 8-day-old culture. Pretreatment, fixation, staining, and magnification same as figure 30.

FIGURE 32.—A polyploid cell in division, 8-day-old culture. Pretreatment, fixation, staining, and magnification same as figure 30.



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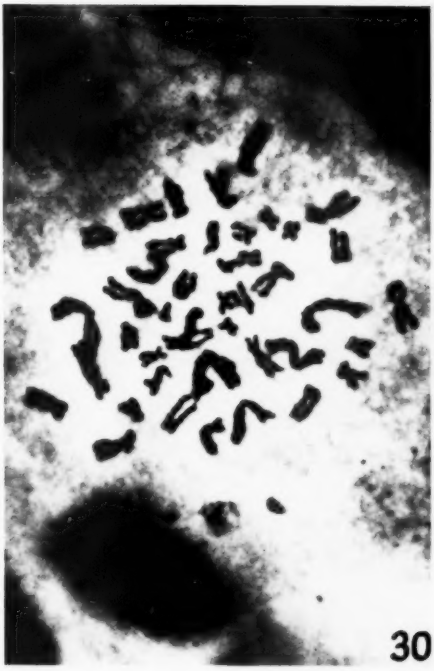

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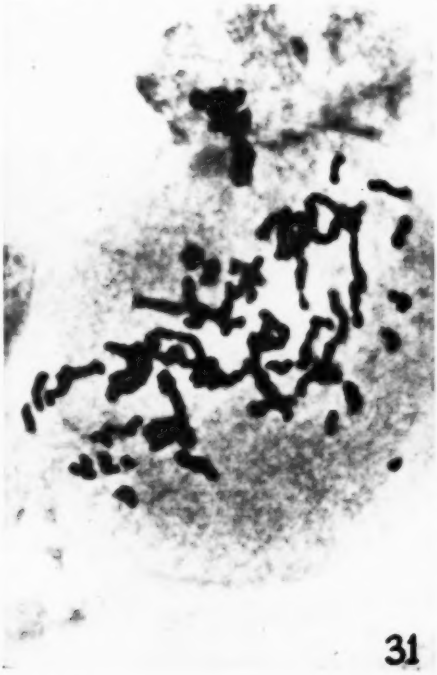

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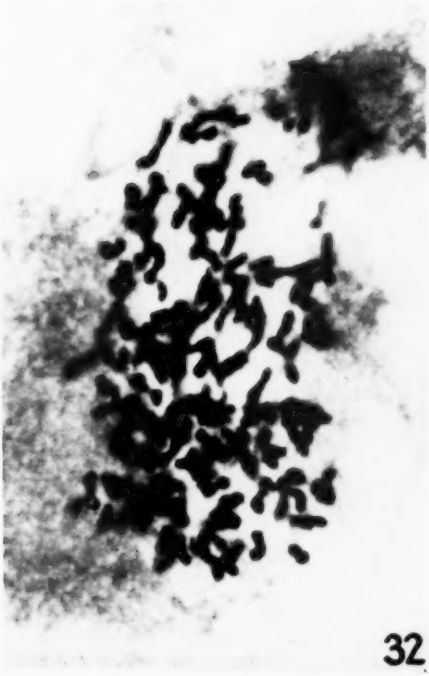

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The Effect of Purines, Nucleosides, and Nucleotides on the Carcinostatic Action of 8-Azaguanine [1, 2]

ALFRED GELLHORN, ERICH HIRSCHBERG, *and* ALICE KELLS, *Departments of Medicine and Biochemistry and the Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, N. Y.*

Since the report of Kidder *et al.* in 1949 (*1*) on the carcinostatic action of 8-azaguanine against several tumors in experimental animals, the compound has been studied extensively in the laboratory and clinic. Although this guanine analog has not proved to be effective in clinical cancer chemotherapy, it continues to be useful as a tool in the investigation of cellular biochemical reactions. For this latter reason a number of studies have been devoted to its mechanism of action. Skipper *et al.* (*2*) demonstrated that treatment of mice with 8-azaguanine caused a decreased rate of incorporation of formate-C^{14} into the nucleic acid purines of viscera. Mitchell *et al.* (*3*) and Heinrich and his associates (*4*) presented evidence for the incorporation of 8-azaguanine into the nucleic acids of mouse viscera and of *Tetrahymena geleii*.

Other investigations on the mode of action of this agent have been based on the antimetabolite theory. The point of departure for these studies was the demonstration by Kidder and Dewey (*5*) of the striking growth-inhibitory effect of 8-azaguanine on *Tetrahymena*, an animal protozoan that requires preformed dietary guanine. This growth-inhibitory effect was reversed by the addition of guanine or guanylic acid to the culture medium. Applying these observations to mammalian cells, Law (*6*) found that the carcinostatic effect of 8-azaguanine against mouse leukemia L1210 was completely reversed by guanine. Definite, though incomplete, release of 8-azaguanine inhibition of mouse mammary adenocarcinoma 755 by guanine was reported by Kidder *et al.* (*7*). Goldin *et al.* (*8*) showed that while guanine produced a minimal reversal of 8-azaguanine-induced inhibition of the growth of three lymphoid mouse tumors, guanylic acid effectively reversed the carcinostatic action of this agent. Guanine decreased the toxicity of 8-azaguanine to chick embryos (*9*) and reversed the inhibitory effect of this agent on the growth of virus tumors of *Rumex acetosa* (*10*). Guanine, as well as adenine, hypoxanthine, and guanosine,

[1] Received for publication October 9, 1953.
[2] This work was supported in part by a grant (C-1386) from the U. S. Public Health Service.

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reversed the inhibitory effect of 8-azaguanine on the multiplication of the lucerne mosaic virus in *Nicotiana glutinosa* and *N. tabacum* (*11*). On the other hand, guanine did not reverse the inhibitory action of 8-azaguanine on crown-gall tumor, excised tomato roots, or sunflower embryos (*12*) or the depressing effect of this agent on antibody production in several strains of inbred mice (*13*).

Investigations on the utilization of exogenous purines for the synthesis of nucleic acids have demonstrated that data obtained in one species or biological system cannot be applied necessarily to another species or system (*14–23*). Hamilton (*24*) recently summarized these species differences in the utilization and interconversion of guanine and adenine in a wide variety of animals and microorganisms. In view of these differences it was deemed of interest to summarize our experiments on the simultaneous administration of 8-azaguanine and various normal constituents of nucleic acids to rabbits bearing the Brown-Pearce carcinoma.

Materials and Methods

The growth characteristics and response to 8-azaguanine of Brown-Pearce tumor transplants in the anterior chamber of the rabbit eye have been described in previous publications (*25*, *26*). The tumors were transplanted into both eyes of each rabbit and were allowed to grow without treatment for 48 to 72 hours after implantation; this permitted adequate time for vascularization of the tumor fragment by vessels from the iris and unequivocal evaluation of the viability of the tumor. Groups of five rabbits each were then treated daily with *a*) physiological saline; *b*) 8-azaguanine at 25 mg. per kg. of body weight; *c*) the potential reversing agent (adenine, guanine, adenosine, guanosine, adenylic acid, or guanylic acid) at 200 mg. per kg. of body weight; *d*) 8-azaguanine at 25 mg. per kg. and the potential reversing agent at 200 mg. per kg., simultaneously. These intraperitoneal injections were continued for 4 to 6 days, until the tumors in the saline control animals had filled the anterior chamber of the eye completely. The tumors were then removed and weighed. Each potential reversing agent was evaluated in at least two separate experiments.

The preparation of the 8-azaguanine solutions has been described previously (*26*). Solutions of the potential reversing agents containing 20 mg. per ml. were prepared as follows: adenine and guanine were placed into one-third the final volume of 10 percent gum acacia, made alkaline with 40 percent NaOH until the solution was clear, and then adjusted to *p*H 8 with 2 N HCl and brought to volume; adenosine was dissolved in hot distilled water; guanosine, guanylic acid, and adenylic acid were dissolved in one-third to one-half the final volume of 0.1 N NaOH with gentle heating and then brought to volume.[3]

[3] The authors are indebted to Dr. J. M. Ruegsegger of the Lederle Laboratories for the supply of 8-azaguanine; batch No. 7-7242 was used in these experiments. The other agents were obtained from Schwarz Laboratories. Ultraviolet spectroscopic analysis indicated a purity of 85 percent for 8-azaguanine and of better than 95 percent for the other agents.

Results and Discussion

Table 1 summarizes the results obtained with the various potential reversing agents. Since in most experiments guanine, adenine, and their ribosides and ribotides produced some inhibition of tumor growth in the dosages employed, the prevention of 8-azaguanine carcinostasis can best be seen in comparisons between the tumor weights of animals treated with the combination of 8-azaguanine plus a nucleic acid constituent, the tumor weights of animals receiving the metabolite alone, and those given 8-azaguanine alone. It is to be noted that, with the exception of guanine, all of the nucleic acid constituents that were tested prevented in part the inhibition of tumor growth by 8-azaguanine.

The lack of effectiveness of guanine in preventing the action of 8-azaguanine on the Brown-Pearce tumor is clear cut; in fact, in 2 of 3 experiments the combination of guanine and its analog produced significantly

TABLE 1.—*Carcinostatic effect of various purines, nucleosides, and nucleotides alone and in combination with 8-azaguanine*

Treatment	Mean tumor weight (*mg.*) ± Standard Deviation*			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Saline	433 ± 98	528 ± 82	532 ± 156	
8-Azaguanine	141 ± 41	314 ± 135	203 ± 70	
Guanine	.....	436 ± 95	404 ± 89	
Guanine + 8-azaguanine	164 ± 44	125 ± 41	176 ± 53	
Saline	353 ± 55	350 ± 81	382 ± 99	423 ± 120
8-Azaguanine	171 ± 49	236 ± 69	151 ± 35	278 ± 99
Adenine	351 ± 69	278 ± 42	305 ± 39	493 ± 210
Adenine + 8-azaguanine	254 ± 49	282 ± 59	283 ± 31	398 ± 105
Saline	627 ± 185	419 ± 123		
8-Azaguanine	149 ± 41	123 ± 61		
Guanosine	459 ± 40	.....		
Guanosine + 8-azaguanine	324 ± 105	239 ± 71		
Saline	330 ± 105	440 ± 79		
8-Azaguanine	143 ± 29	172 ± 39		
Adenosine	368 ± 110	399 ± 82		
Adenosine + 8-azaguanine	310 ± 119	384 ± 88		
Saline	355 ± 46	433 ± 98	523 ± 115	
8-Azaguanine	163 ± 53	141 ± 41	173 ± 63	
Guanylic acid	.....	.....	390 ± 46	
Guanylic acid + 8-azaguanine	251 ± 93	293 ± 126	294 ± 87	
Saline	474 ± 52	698 ± 133	675 ± 207	
8-Azaguanine	124 ± 26	245 ± 75	212 ± 63	
Adenylic acid	388 ± 64	490 ± 130	500 ± 176	
Adenylic acid + 8-azaguanine	234 ± 47	622 ± 153	428 ± 125	

Note: 8-Azaguanine was administered at 25 mg. per kg. of body weight, the potential reversing agents at 200 mg. per kg. The mean tumor weight for each group is the mean of a group of 10 tumors in 5 animals.

*Standard Deviation $\sigma = \sqrt{\frac{\Sigma d^2}{n-1}}$

greater tumor inhibition than the analog alone. In the other experiments, adenine and its nucleoside and nucleotide were appreciably more active than guanosine and guanylic acid as inhibitors of 8-azaguanine carcinostasis. It should be stressed that the difference in tumor weights between groups treated with 8-azaguanine and with the combination was statistically significant in 13 of 14 experiments.

These results are at variance with the findings of other investigators (*5–11*) who demonstrated a metabolite-antimetabolite relationship between guanine and 8-azaguanine in other biological systems. On the other hand, the significantly greater effectiveness of guanylic acid in comparison to guanine reported by Goldin *et al.* (*8*) in mouse tumors is also seen in this rabbit neoplasm. In addition, data are presented here on the anti-azaguanine activity of other compounds which are part of the fundamental pathway of nucleic acid synthesis.

In view of the greater effect of nucleosides and nucleotides in decreasing the carcinostatic activity of 8-azaguanine, it is tempting to postulate that the analog must be transformed into a riboside or ribotide before having its effect; a transpurination reaction with 8-azaguanine has been described by Friedkin (*27*). The appreciably greater reversing effect of adenine and its derivatives and the lack of any such effect of guanine might be interpreted to indicate a conversion of guanine to adenine prior to riboside formation and an inhibition of this conversion by 8-azaguanine. In the absence of any information on the actual pathways of incorporation of purines into nucleic acids of the Brown-Pearce tumor, these interpretations are speculative.

Summary

The carcinostatic activity of 8-azaguanine (25 mg. per kg. of body weight) against the Brown-Pearce carcinoma growing in the anterior chamber of the rabbit eye was significantly decreased by the simultaneous administration of adenylic acid, adenosine, adenine, guanylic acid, and guanosine (200 mg. per kg. each). Guanine (200 mg. per kg.) not only did not decrease the carcinostatic activity of 8-azaguanine against this tumor, but actually appeared to potentiate it.

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The Effect of X Irradiation and Lowered Metabolic Rate on the Morphogenesis of Developing *Melanoplus differentialis* Embryos [1]

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Previous communications have indicated that X irradiation of diapause grasshopper embryos which were kept at 25° C. following exposure resulted in regression in size, coupled with an increase in hydroquinone oxidase (*1*, *2*). X irradiation rendered practically every nucleus in the embryo pyknotic, but the respiratory metabolism of the embryos undergoing regression was not abolished; in point of fact, some acceleration in oxygen utilization was observed. This phenomenon was ascribed to the inability of the nuclei to maintain their integrity by forming specific proteins for embryonic cells from the surrounding yolk deposits. The increase in hydroquinone oxidase activity was attributed to the shunting of anabolic processes to the catabolic cycle, since catabolism was found to be more resistant to X irradiation than anabolism.

Normally, every cell in the diapause embryo is in the resting stage. The metabolism is of basal order, and the embryo neither grows nor regresses. If diapause is interrupted by cold treatment, to simulate winter, the embryos grow by mitosis and tissue differentiation and hatch 17 days after they are placed at 25° C. from cold treatment at 2° ± 1° C.

Recently Boell (*3*) described the role that metabolism plays as an indicator of irradiation damage in insects. Because sensitivity to irradiation seems to depend on metabolism, experiments have been carried out in this laboratory to study the effect of altered metabolic rate on the radiosensitivity of grasshopper embryos at the time of irradiation. In addition, morphologic and physiologic changes in the fundamental processes of cell division and tissue differentiation have been observed. These are believed to be pertinent to a better understanding of the response of malignant tissue to irradiation and its relationship to the normal tissue surrounding it.

Materials and Methods

Egg pods of *Melanoplus differentialis* were collected daily from an indoor colony, placed on moist, synthetic sand, and kept at 25° C. ± 0.05° C. for

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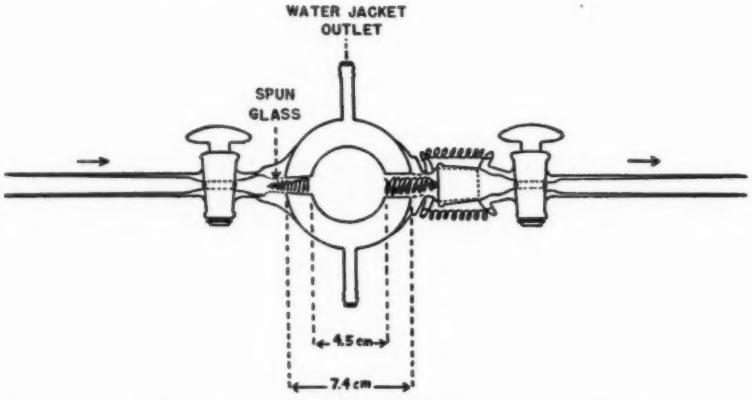
30 days, at which time they have entered diapause (*4*). They were removed from the pods and placed in 3 percent NaOCl (*5*) for 5 minutes, until the chorion dissolved and the embryos could be seen through the cuticles. They were then rinsed, first with glass-distilled water, then with 3 percent citric acid to neutralize the NaOCl, and finally several times with pyrogen-free double-glass-distilled water. The size of the embryo and the presence of an intact amnion served as criteria for selecting eggs in which diapause was unbroken. The eggs were then treated with water saturated with Moldex[4] (*n*-butyl-parahydroxy benzoate) and approximately 8,000 eggs were placed in each of ten 5-cm. petri dishes. Eggs that were to be gassed, irradiated, or gassed and irradiated, were transferred from the petri dishes into a specially designed, temperature-controlled vessel for experimentation (text-fig. 1). The temperature in this vessel was maintained at 25° C. ± 0.05° C. by pumping water through the glass jacket surrounding the eggs.



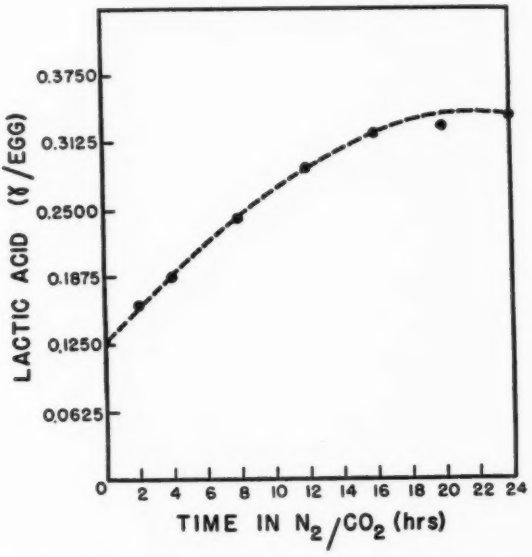
TEXT-FIGURE 1.—Pyrex gassing chamber in which eggs were irradiated at a constant temperature, which was controlled by the outer water jacket.

The gas used for anaerobiosis was a mixture consisting of 95 percent N_2 and 5 percent CO_2 (hereafter, N_2/CO_2 will indicate this mixture), which was depleted of oxygen by passage over reduced copper shavings heated to 800° C. The 5 percent CO_2 was added to maintain a low internal *p*H, which counteracts radiation protection of tissues at higher *p*H values (*6–8*). The gas was cooled and saturated by bubbling through water before delivery to the eggs. At the exhaust another bubbler served as an air trap.

Petri dish #1 was kept as control; #2 was irradiated at 2,500 r in air; #3 was irradiated at 25,000 r in air; #4 was placed under N_2/CO_2 for 24 hours and kept as control; #5 was treated like #4 but at various intervals (text-fig. 2) for 24 hours samples were withdrawn for lactic acid determination, which is discussed below. Sample numbers 6, 7, 8, 9, and 10 were

[4] General Biological Supply Company, Chicago, Ill.

irradiated at 25,000, 120,000, 240,000, 500,000, and 980,000 r under anaerobiosis. Three experiments with grasshopper eggs divided as described above (in petri dishes 1 through 8) were repeated three times consecutively. Experiments with the contents of petri dishes 9 and 10 were not repeated because nearly complete inhibition of biologic processes resulted from these high dosages.



TEXT-FIGURE 2.—Increment of lactic acid over a period of 24 hours. 0 time is the same as control or aerobic lactic acid level of embryos.

Irradiation was accomplished with a 15-ma., 200-kv. machine, the dose rate being 1,131 to 1,331 r per minute without filters except for 2 layers of 1-mm. thickness of pyrex glass surrounding the eggs in the special chamber.

The effect of irradiation on hydroquinone oxidase was determined daily with the aid of Warburg manometers according to the method previously described (*2*).

The initial and final lactic acid content of the anaerobic eggs was determined in quadruplicate by Barker and Summerson's method (*9*). The standard lactate solutions were prepared from recrystallized lithium lactate,[5] and a Beckman DU spectrophotometer was used for the determinations at 565 μ.

Some of the eggs irradiated at 2,500 and 25,000 r in air and 25,000, 120,000, and 240,000 r under N_2/CO_2 were placed at $2° \pm 1°$ C. for three months to interrupt diapause (*4*). These cold-treated eggs were then returned to $25° \pm 0.05°$ C. and daily morphologic and cytologic studies were made.

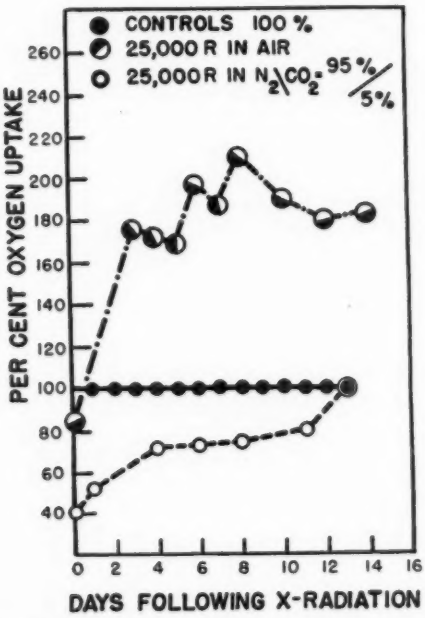
[5] We are indebted to Dr. John H. Pomeroy of this division for recrystallizing this compound.

Other experimental material was fixed in Allen's B15 solution, embedded in paraffin, sectioned at 7 μ, and stained with Heidenhain's iron hematoxylin or Flemming's triple stain as modified by Foley (*10*). Random samples of the remaining material were studied *in vivo* with a Leitz phase-contrast optical system in Belar solution.

Results

Regression in size.—Regression in size from the diapause level is caused by irradiation and pyknosis of the embryonal nuclei without respiratory inhibition. [*Cf.* fig. 1C, normal postdiapause embryo, with 1E which shows regression in size (*1*).] Under aerobic conditions, grasshopper eggs that were exposed to 25,000 r underwent regression (*1*), while under anaerobiosis a dose of 240,000 r was required to produce this effect [fig. 1E, also see (*4*).] Regression in size did not result when eggs were irradiated at 25,000 r after 22 to 24 hours of N_2/CO_2 atmosphere (fig. 1D). Exposure to 25,000 r in N_2/CO_2 resulted in inhibition of tissue differentiation as did 2,500 r in air.

Enzymes.—Six to 14 days after irradiation there was an increase in hydroquinone oxidase in eggs exposed to 25,000 r in air. The same dose given at the end of 24 hours of anaerobiosis effected a drop in the enzyme activity, but after 14 days the hydroquinone content rose to the level of that found in controls (text-fig. 3). This drop in the hydroquinone



TEXT-FIGURE 3.—Hydroquinone oxidase levels over a period of 14 days postirradiation as indicated.

oxidase is attributed to 24-hour treatment with N_2/CO_2 and not to X irradiation, because nonirradiated controls similarly treated show a 33 percent drop (table 1). Although some increase in enzymatic activity occurred with 120,000 r, in order to elicit an increased activity comparable with that occurring in eggs irradiated in air, 240,000 r was necessary (table 1).

TABLE 1.—*Enzymatic activity of hydroquinone oxidase (hydroquinone 0.033 M, pH 7.4, PO_4–NaOH buffer in H_2O cytochrome C final concentration 2.4 × 10^{-4}M)*

Treatment	mm.^{3} O_2 per hr.	Percent of control
Control air	306	100
2,500 r air	305	100
25,000 r air	565	184
Control N_2/CO_2 (95%/5%)	219	100
25,000 r N_2/CO_2 (95%/5%)	210	95
120,000 r N_2/CO_2 (95%/5%)	313	142
240,000 r N_2/CO_2 (95%/5%)	452	206

Free energies.—When the metabolic rate was lowered, a higher radiation dose was necessary to cause radiation damage equal to that which occurred under normal metabolic conditions. Under aerobiosis a grasshopper embryo utilizes 0.25 cu. mm. of O_2 per egg per hour at 25° C. At this rate all of the oxygen dissolved within the egg would be used up in 5 minutes if the egg was removed from an atmosphere containing oxygen. Under anaerobic conditions in an atmosphere of N_2/CO_2 over a period of 24 hours, the initial lactic acid formation was 0.016 γ per egg per hour, decreasing to 0.003 γ per egg per hour at 16 hours (text-fig. 2). If we assume that the initial metabolite is glucose, the amounts of the CO_2 production aerobically or lactic acid formation anaerobically per egg per hour represent 1.8×10^{-9} M and $.07 \times 10^{-9}$ M of glucose respectively. Knowing the ΔF of 689 k calories for the CO_2 and H_2O production and 60.16 k calories for glucose degradation to lactic acid from the amounts indicated above, we find that there is a lowering of metabolism about 300 times under anaerobic conditions. Under these conditions, 10 times the radiation dose was necessary to elicit the same results obtained under aerobic conditions.

Morphogenesis.—When eggs were irradiated at 2,500 r in air or 25,000 r under N_2/CO_2 while in diapause and placed at 2° C. ± 1° C. for 3 months to interrupt diapause, they grew when they were returned to developmental temperature at 25° C. During this postdiapause development their external morphology (fig. 1D) eventually resembled that of a 12- to 14-day postdiapause embryo, but they developed no further although they were kept at 25° C. for 38 days, or 20 days beyond the time at which a normal embryo hatches. No normal chromosomes were seen, either under living conditions (figs. 2, 3), or in fixed cells (figs. 4 to 9). Although cell division was abundant even after 38 days postdiapause, tissue differ-

entiation did not occur in embryos that received 2,500 r in air or 25,000 r under N_2/CO_2 (fig. 9). In a few embryos the only indication of functional differentiation was the deposition of cuticle by the ectodermal cell layer representing the exoskeleton (fig. 9). A comparison of figure 9 with control figures 10 and 11 indicates that the induction of organ systems such as malpighian tubules (fig. 10), striated muscle (fig. 11), trachea (fig. 11), and connectives and commissures of the nervous system is abolished in the irradiated material.

Eggs receiving 25,000 r in air or 240,000 r under N_2/CO_2, whether left in diapause or in postdiapause following cold treatment, developed pyknotic nuclei by 16 days of developmental temperature following irradiation (fig. 12) which resulted in regression in size.

Discussion

In the normal development of an embryo there is a definite sequence of biological processes, *i.e.* metabolism, cell division, and tissue differentiation, which culminates in the formation of the animal. The susceptibility of these fundamental processes to X-radiation damage is also sequential; however, the order is reversed.

These experiments indicate that the capacity of tissues to differentiate physiologically is the process most sensitive to X irradiation. The next stage in the order of sensitivity is the ability of the cell to divide.

In a personal communication to the senior author, Dr. Roberts Rugh stated that in amphibia he found the stage occurring just prior to morphogenesis to be the most susceptible to radiation injury. In two previous communications (*1*, *2*) it was shown that anabolism is more susceptible to X radiation than catabolism. Under aerobiosis the normal energy metabolism ends with the production of CO_2 and H_2O, with a definite caloric output in free energy. Under anaerobic conditions the formation of lactic acid yields a definite caloric output per mole of substrate used. We have assumed that the original substrate is glucose. From the metabolic end products under aerobiosis and anaerobiosis, it was found that the amount of glucose used in a given time interval varied with the metabolic rate. More glucose was used under aerobic conditions, and there was much more free energy available per mole of glucose degradation. Under anaerobic conditions 26 times less glucose was used in a given time, and for a period of up to 24 hours the metabolic rate was lowered about 300 times below the aerobic level.

One of the purposes of this study was to investigate the effect of lowered metabolic rate on the radiosensitivity of cells. It was found that when metabolism was lowered under anaerobiosis, a ten times greater X-ray dose was required to produce the same degree of damage as that found under normal aerobic conditions. We regard as significant the observation that the susceptibility of a given cell to irradiation is more dependent upon metabolic rate than upon any other factor at the time of exposure.

Also significant is the finding that for doses of equivalent effectiveness, the physiologic, morphologic, and cytologic damage is identical. Physi-

ologic examination of diapause embryos exposed to 2,500 r in air or 25,000 r under N_2/CO_2 revealed that postdiapause development proceeded more slowly than in control embryos, but the tissues did not differentiate. From a cytologic point of view, cell division was uninterrupted, but as the photomicrographs illustrate, there was not a single cell with a normal chromosomal configuration. The large number of cells in division suggested that the normal duration of the mitotic cycle was increased. Morphologic observations revealed that the embryos grew but did not hatch even after 20 days beyond the normal 18-day hatching time of postdiapause development.

When diapause embryos were given 25,000 r in air or 240,000 r under N_2/CO_2 and left in diapause at room temperature (25° C.), every nucleus became pyknotic (*1*) and regression in size of the embryo occurred, with some increase in respiration. If the embryos were irradiated and placed at 2° C. for 3 months to interrupt diapause and then returned to 25° C. for postdiapause development, they revealed no X-radiation effects immediately. By the 16th day postdiapause at 25° C. there was regression in size equal to that described above. With cold treatment the effect of X radiation was kept latent for 3 months, suggesting that metabolic processes were the "developer" of irradiation injury.

Although Cook found that cold treatment protected *Ascaris* eggs against X-ray damage (*11*), we have found no similar protection with diapause grasshopper eggs, even when they were subjected to cold treatment (2° C.) for 3 to 7 months.

The relative susceptibilities of normal cell processes and of the sequential processes in embryonal tissues have certain implications for the study of malignant tissues. It is obvious that malignant tissues, like embryonal cells, undergo division. If such tissue is irradiated, a given dose will stop cell division without interfering with either anabolism or catabolism, and the malignancy will remain static. However, redifferentiation to normal tissue cannot occur because the capacity for differentiation is more sensitive to X radiation than are other fundamental processes. A higher dose may inhibit anabolism and bring about the regression of the tumor through its inherent catabolic or energy metabolism. Should cells of normal tissue surrounding the irradiated tumor begin to divide, they may be unable to stop dividing. As a consequence, there may be *de novo* formation of malignant tissue at or near the site of the original tumor.

One explanation of radiation-induced tumors may be that the capacity of tissue to continue differentiation or to redifferentiate is inhibited; therefore, when normal tissue is sufficiently irradiated and begins cell division, it may not be able to undergo induction into its own normal function, and a tumor may be formed.

Summary

The extent of X-ray injury in developing grasshopper embryos was determined by cytologic, morphologic, and physiologic examination. At an optimum dose of X radiation the capacity of the cells to maintain

organization was abolished, and they underwent regression in size. Under anaerobic conditions ten times the X-ray dose was required to elicit the same degree of damage observed aerobically, while the caloric output under aerobic conditions was about 300 times that measured anaerobically. The sequence of biological susceptibility to X irradiation is: tissue differentiation>cell division>anabolism>catabolism.

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PLATES

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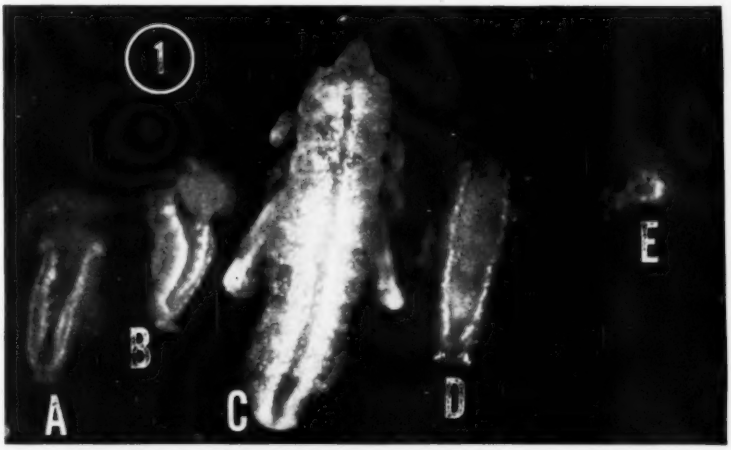
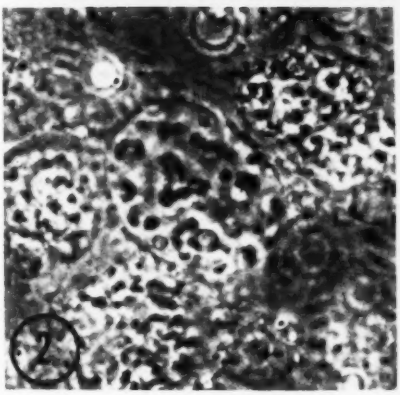
FIGURE 1.—Left to right: *A*) diapause embryo irradiated at 980,000 r under N_2/CO_2, photographed at 14 days postdiapause stage. *B*) diapause embryo irradiated at 500,000 r under N_2/CO_2, 14 days postdiapause development. *C*) control embryo N_2/CO_2-treated, not irradiated, 14 days postdiapause embryo. *D*) diapause embryo irradiated at 25,000 r under N_2/CO_2, photographed on the 14th day of postdiapause development. *E*) diapause embryo irradiated at 25,000 r in air, photographed at 14 days postdiapause development. *Note:* Diapause embryo irradiated at 2,500 r in air appears like *D* above.

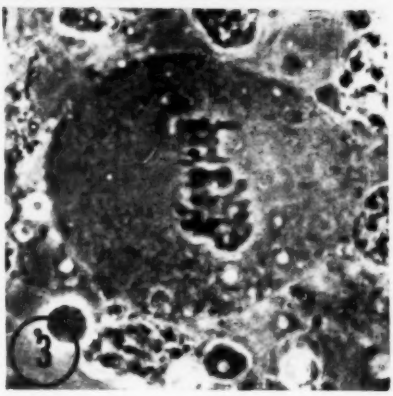
FIGURE 2.—Embryos irradiated under N_2/CO_2 at 25,000 r during diapause, showing mitosis in living embryonic cells during postdiapause development. Lampbrush chromosomes showing translocations. Phase-contrast microscopy in Belar solution of living cells. × 1,800

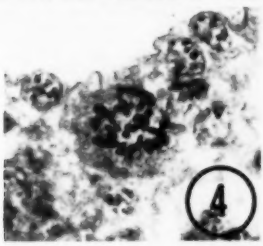
FIGURE 3.—Phase-contrast photomicrograph of living embryonic cells in postdiapause stage that were irradiated under N_2/CO_2 at 25,000 r during diapause. Metaphase showing sticky chromosomes, spindle area, and numerous mitochondria. × 1,800

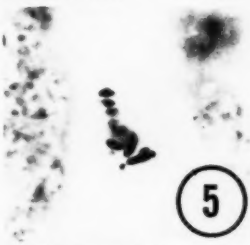
FIGURES 4 to 8.—Cells irradiated in N_2/CO_2 during diapause and fixed at 8 days postdiapause development, showing early stages of mitosis, fewer than the 24 somatic number of chromosomes, and chromosome aberrations, breaks, and crossovers. × 970

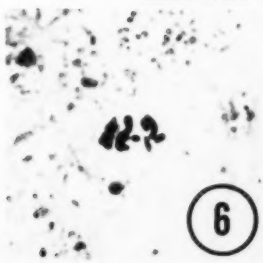


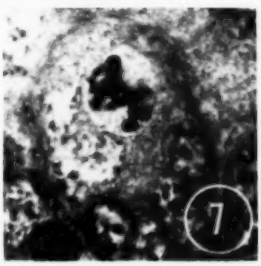












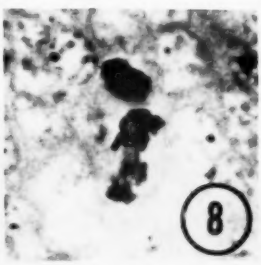


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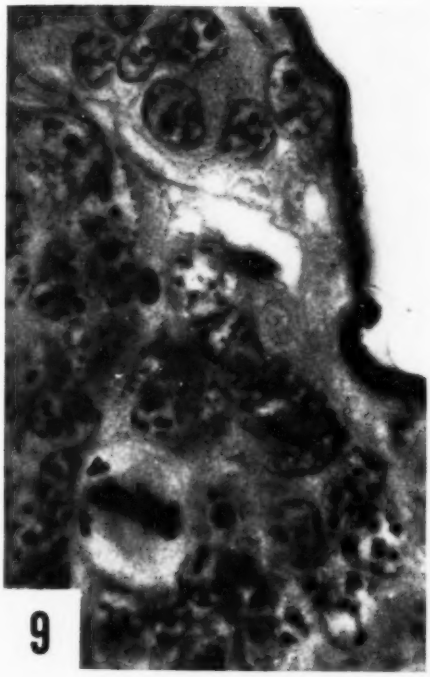
PLATE 78

FIGURE 9.—Section through 16-day postdiapause embryo irradiated at 25,000 r during diapause stage under N_2/CO_2, showing intact cells and mitoses. (*Note:* 2,500 r in air caused identical effects.) × 970

FIGURES 10 and 11.—Sections of control 16-day postdiapause embryos showing malpighian tubules, and striated muscle, tracheole and apodemes, respectively. × 970

FIGURE 12.—Section through 16-day postdiapause embryo irradiated at 240,000 r while in diapause under N_2/CO_2, showing pyknotic nuclei. × 480



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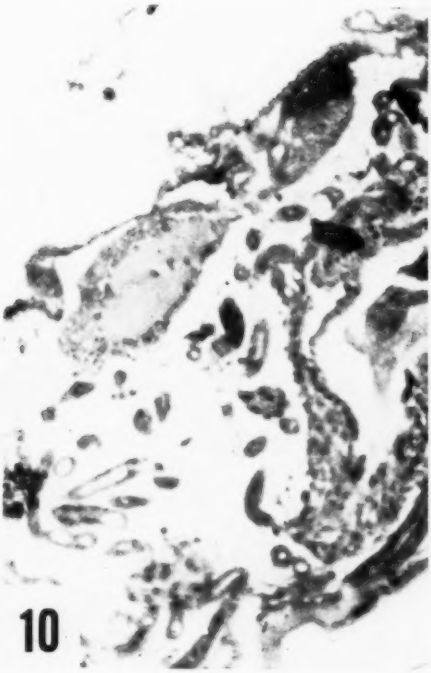

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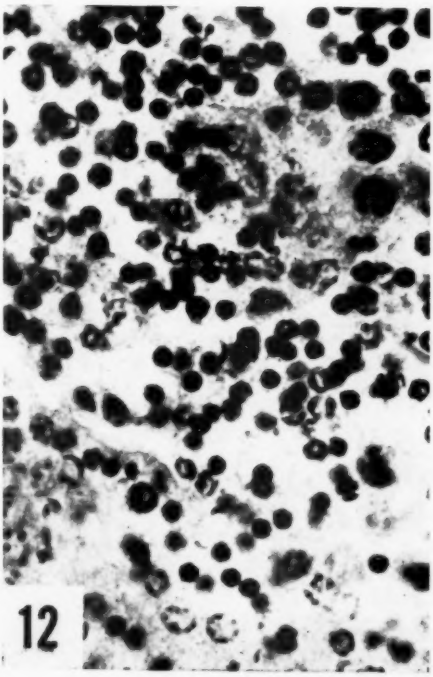

12

Modification of Lethal Irradiation Injury in Mice by Injection of Homologous or Heterologous Bone [1]

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In previous experiments (*1–4*) it was shown that injection of homologous or heterologous bone marrow protected mice against a lethal dose of X radiation. The injection of bone marrow was always highly effective when it was given intravenously, while intraperitoneal injection was with few exceptions considerably less effective. The reason for this decreased effectiveness was found in serial studies of irradiated guinea pigs into which bone marrow was transplanted intraperitoneally. At autopsy, nodules of the bone-marrow transplant were found attached to the omentum. Histologically (*3*) these nodules showed the following series of events: A few days after irradiation, most of the transplant consisted of dead bone-marrow cells. Four to five days after irradiation, formation of trabeculae of osteoid tissue took place. At about the sixth day, blood-forming cells began to appear between the trabeculae, and in succeeding days these greatly increased in amount. At the time blood-forming cells were observed in the transplants, regeneration of the bone marrow of the host also took place. It was not possible to learn whether the bone marrow found in the transplant arose from a few cells that stayed alive after transplantation or whether this marrow had its origin from the bone forming in the transplant. To test the possibility that bone transplants may stimulate hematopoiesis, thus protecting the host from lethal irradiation injury, or that bone itself has protective qualities, experiments were undertaken in which bone was transplanted into irradiated mice. They showed that bone has protective properties and are reported in this paper.

Experimental Procedures

The animals used in the experiments were the homogeneous LAF_1 hybrids and strain A mice. They were irradiated at approximately 3 months of age with a single total-body tissue dose of 800 r or 900 r. These doses are usually lethal within 21 days. To obtain uniform tissue doses, two tubes opposite each other were used; their foci were 54 cm. from the center

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of the mice. The tubes were operated at 186 KVP and 20 ma.; beam filtration was 0.25 mm. copper and 1.0 mm. aluminum. The mice were irradiated in shallow cylindrical boxes holding 10 mice, and the tissue dose rate was 100 r per minute ±5 percent under these conditions. The changes in dose rate were caused by several tube replacements after the experiments were started.

One or two hours after irradiation, the mice were injected intraperitoneally or subcutaneously with the bone preparation. The bone was obtained from approximately 1-month-old mice or rats. The femurs and humeri were removed, the ends cut off, and the shafts cleaned of adhering tissue. Then they were split open, the bone marrow removed by scraping or brushing, and the bone cut into small pieces for intraperitoneal injection by trocar. The cleaned bone pieces were kept moist with buffered saline (*5*). Since in the first experiments a few mice were lost by bone pieces puncturing the intestine, in later experiments the bone pieces were worked over in buffered saline in a mortar. Although this procedure did not break up the pieces of bone, sharp edges were sufficiently rounded to prevent piercing of the intestinal wall. Samples of these pure white pieces of bone were examined histologically (fig. 1) and rarely showed a microscopic focus of bone marrow. Finally, a bone grinder was constructed which consisted of a heavy stainless steel cylinder of 3 cm. inner diameter with a removable bottom. The surface of the bottom had milled grooves, approximately 0.25 mm. in depth, at right angles. A plunger, the bottom of which was milled with parallel grooves, 1 mm. apart and 0.25 mm. deep, fitted loosely into the cylinder. It was driven by a drill press at slow speed. Cleaned pieces of bone were placed in the cylinder with a few milliliters of buffered saline. A few up-and-down movements of the rotating plunger effectively ground the bone to particle sizes of a small fraction of a millimeter. In some experiments, the bone particles were centrifuged and the bone brei injected by trocar; in others it was kept in suspension by frequent shaking and injected through an 18-gauge needle. Usually approximately 40 mg. of bone were injected.

Attempts to prepare a bone suspension suitable for intravenous injection have failed so far. The bone, ground as described above, was further ground in a Ten Broeck homogenizer. However, agglutination of the particles caused emboli after intravenous injection, even if the amounts injected were decreased to approximately 1 mg. The bone callus used in experiment III, table 1, was obtained by removing the fracture site from the tibia and fibula about two weeks after the injury, and working it over in a mortar.

In most experiments autopsies were performed on representative animals that were found dead and on animals sacrificed at the termination of the experiment.

Results

The effect of injection of homologous bone is shown in table 1. A survival period of 21 days was chosen, since in that time all control mice died. Later death occurred only occasionally. All surviving animals

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TABLE 1.—*Survival of LAF_1 mice injected with homologous bone following irradiation*

Exp. No.	Dose (*r*)	Treatment	Number of mice	Number of mice dying each day after exposure																Mortality (*percent*)
				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16–21	
I	800	none	21	–	–	–	–	–	–	1	–	5	5	4	3	1	1	–	1	100
II	800	pieces of bone, intraperitoneally	20	–	–	–	–	1	2	–	–	–	–	1	1	2	2	–	1	50
III	800	pieces of bone callus, intraperitoneally	12	–	–	–	–	–	–	–	–	–	–	–	2	2	–	–	–	33
IV	900	none	20	–	–	–	–	–	–	5	6	2	1	3	1	1	1	–	–	100
V	900	pieces of bone, intraperitoneally	19	–	–	–	–	–	–	–	–	–	2	1	–	2	1	–	–	32
VI	900	ground bone, intraperitoneally	19	–	–	–	–	–	–	1	–	–	–	–	–	–	–	–	2	16
VII	900	ground bone, subcutaneously	13	–	–	–	4	–	–	–	–	–	1	–	4	–	–	–	1	77

were sacrificed 30 to 60 days after irradiation for histologic studies. The data show that pieces of bone injected intraperitoneally by trocar give good protection (experiments II and V).

Autopsy of animals that did not survive the 21-day period showed failure of the hematopoietic tissues to regenerate. The pieces of transplanted bone showed survival of many osteocytes and patches of hematopoiesis in the fibrous connective-tissue reaction of the host to the pieces of bone. In animals surviving the 21-day period the pieces of bone showed rearrangement of the bone spicules into small masses of bone with bone-marrow formation in the marrow spaces (fig. 2). The hematopoietic tissues of the host showed complete regeneration.

Bone callus obtained from mice approximately 2 weeks after their tibia and fibula were broken and transplanted intraperitoneally into irradiated mice improved survival considerably compared with the untreated irradiated controls (experiment III). The pieces of bone callus consisted of osteoid, cartilage, bone, and fibrous connective tissue. Autopsy of animals that did not survive the 21-day period showed survival of the transplanted callus and patches of blood-forming cells among the elements of the transplanted callus. In the animals living beyond the 21-day period the transplanted callus showed reorganization into nodules of bone and bone marrow. This was essentially the same process as was observed when pieces of cortical bone were transplanted into irradiated mice.

The results obtained with ground bone brei given intraperitoneally or subcutaneously are shown in experiments VI and VII.

Similar experiments were done using bone of young rats as the donor tissue for irradiated LAF_1 and strain A mice. They are listed in table 2. Pieces of bone or ground bone transplanted intraperitoneally (experiments 2, 3, and 8) protected LAF_1 mice. On the other hand, rat bone transplanted intraperitoneally into irradiated strain A mice (exp. 5) gave no protection, which is consistent with the previous observation that intraperitoneally transplanted homologous bone marrow gave little protection in this strain against irradiation injury (*2*). No protection either in LAF_1 mice or strain A mice was obtained by intravenous injection of the supernatant of centrifuged ground bone (experiments 6 and 9).

The pieces of rat bone injected were histologically similar to those of mouse bone shown in figure 1. Microscopic foci of bone marrow in these pieces of cortical bone were extremely rare. The pieces of rat cortical bone in the irradiated mice that did not survive the 21-day period showed encapsulation by fibrous connective tissue. There was no definite evidence of blood formation and most of the osteocytes had disappeared. Some of the transplants recovered from mice dying during the 21-day period showed slight bone proliferation. However, the hematopoietic tissues of these nonsurvivors failed to regenerate. In animals surviving beyond the 21-day period following total-body irradiation, the hematopoietic tissues had regenerated. The transplants in these surviving mice consisted of necrotic pieces of cortical bone encapsulated in fibrous connective tissue with varying degrees of foreign-body giant-cell reaction

TABLE 2.—*Survival of mice injected with rat bone following irradiation*

Exp. No.	Dose (r)	Donor rat strain	Treatment	Recipient	Number of mice	Number of mice dying each day after exposure															Mortality (*percent*)
						1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16–21	
1	800	none	none	LAF_1	21	–	–	–	–	–	–	1	–	5	5	4	3	1	1	–	1	100
2	800	Fisher	pieces of bone, intraperitoneally.	LAF_1	10	–	–	–	–	–	–	–	–	–	–	4	–	1	–	–	–	50
3	800	Osborne-Mendel	ground bone, intraperitoneally.	LAF_1	20	–	–	–	–	–	–	–	1	–	1	1	2	–	–	2	6	65
4	900	none	none	A	35	–	–	–	1	3	12	1	3	2	7	5	–	–	1	–	–	100
5	900	Osborne-Mendel	pieces of bone, intraperitoneally.	A	20	–	1	–	4	5	3	5	2	–	–	–	–	–	–	–	–	100
6	900	Osborne-Mendel	supernatant of ground bone, intravenously.	A	10	–	–	–	–	–	–	2	–	2	2	1	3	–	–	–	–	100
7	900	none	none	LAF_1	20	–	–	–	–	–	–	5	6	2	1	3	1	1	1	–	–	100
8	900	Osborne-Mendel	ground bone, intraperitoneally.	LAF_1	8	–	–	–	–	–	–	1	–	1	2	1	–	–	–	–	–	62
9	900	Osborne-Mendel	supernatant of ground bone, intravenously.	LAF_1	8	–	–	–	–	–	–	1	–	–	5	1	1	–	–	–	–	100

(fig. 3). No definite evidence of blood formation in the transplant was observed.

The ground brei of rat bone consisted of microscopic fragments of bone containing one or a few osteocytes per fragment and abundant bone debris. In surviving irradiated mice this material was encapsulated in the abdominal cavity by dense fibrous connective tissue. The microscopic bone fragments were necrotic and there was no evidence of blood formation in relation to the transplant. Figure 4 shows the type of reaction observed. The hematopoietic organs in the surviving animals showed regeneration, but in nonsurvivors this regeneration was not observed.

Discussion

The data presented show that bone protected mice against lethal irradiation injury in a manner comparable to the protection afforded by bone marrow. It could be argued that the protection by bone was caused by islands of bone marrow remaining in spite of careful cleaning of the bones. Indeed, such microscopic islands were found in samples of the bones used for transplantation. However, the amount of bone marrow obtainable from the femurs and humeri of 1-month-old mice is only 1 to 1.5 mg. of wet tissue. After cleaning the marrow from the bone, only rare microscopic foci of marrow remain and it seems very doubtful that such a small amount would afford a protection similar to that obtained from 1 to 1.5 mg. of marrow tissue given intraperitoneally. In the days following irradiation, bone-marrow formation was observed in homologous transplants of nonsurvivors and was present in the surviving animals autopsied 30 to 60 days after irradiation. The impression gained was that the bone should be considered the precursor of the newly formed bone marrow. A similar conclusion can be made from the experiments of Heinen (*6*) who showed that outgrowths of bone in tissue culture, on transplantation back to a homologous host, differentiated into bone and bone marrow. Therefore, if we assume the existence of humoral factors in irradiation protection by spleen shielding and bone-marrow injection as postulated first by Jacobson (*7*), osseous tissue should also contain such a factor. This factor may be the same as in bone-marrow protection. The experiments in which it was shown that rat bone also affords protection against irradiation injury, give additional proof that bone may contain such a factor. The histologic evidence shows that rat bone transplanted into irradiated mice does not form bone marrow, yet it protects irradiated mice. Recently (*8*) it has been shown that bone marrow from rats protects mice against total-body irradiation injury.

Conclusions

Bone of mouse and rat when transplanted intraperitoneally protects LAF_1 mice from a lethal dose of X radiation.

Homologous bone induced bone-marrow formation in the transplant; it is postulated that this bone marrow originates from the osseous tissue.

Heterologous rat bone protects LAF_1 mice also but bone-marrow formation did not occur in the transplant.

The experiments give further evidence for the existence of a humoral factor or factors in postirradiation protection.

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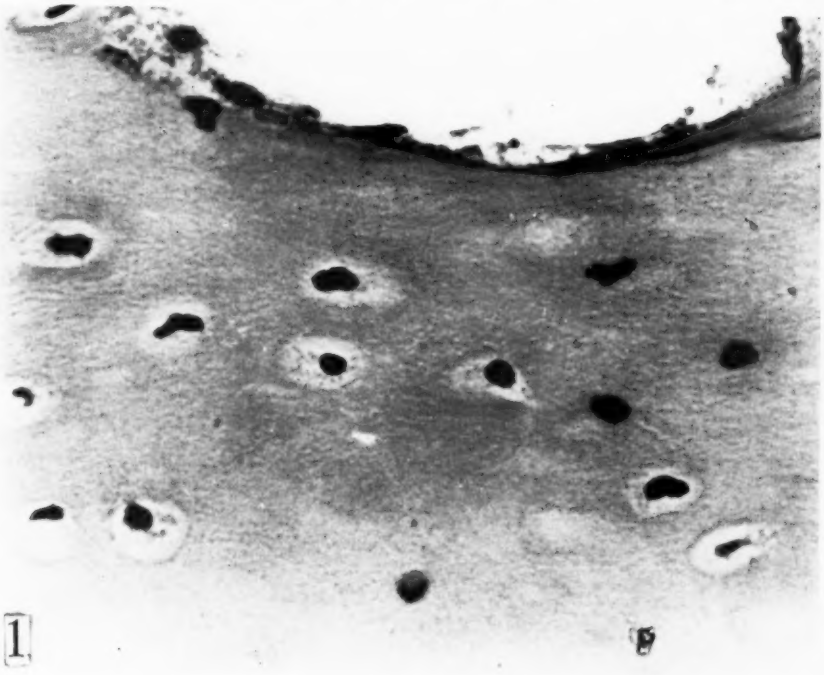
PLATE 79

FIGURE 1.—Piece of cortical bone from a mouse illustrating the type of tissue injected in most of these experiments. The space is a haversian canal. This specimen was prepared by working the pieces of bone freed of marrow in saline in a mortar. Hematoxylin and eosin. × 380

FIGURE 2.—Transplant of mouse bone recovered from the peritoneal cavity of a mouse 22 days following 800 r total-body irradiation and an intraperitoneal injection of cortical bone. The original bone injected resembled that shown in figure 1. Note that many of the osteocytes have survived and that the reorganization of the bone has produced marrow spaces filled with red bone marrow. Hematoxylin and eosin. × 80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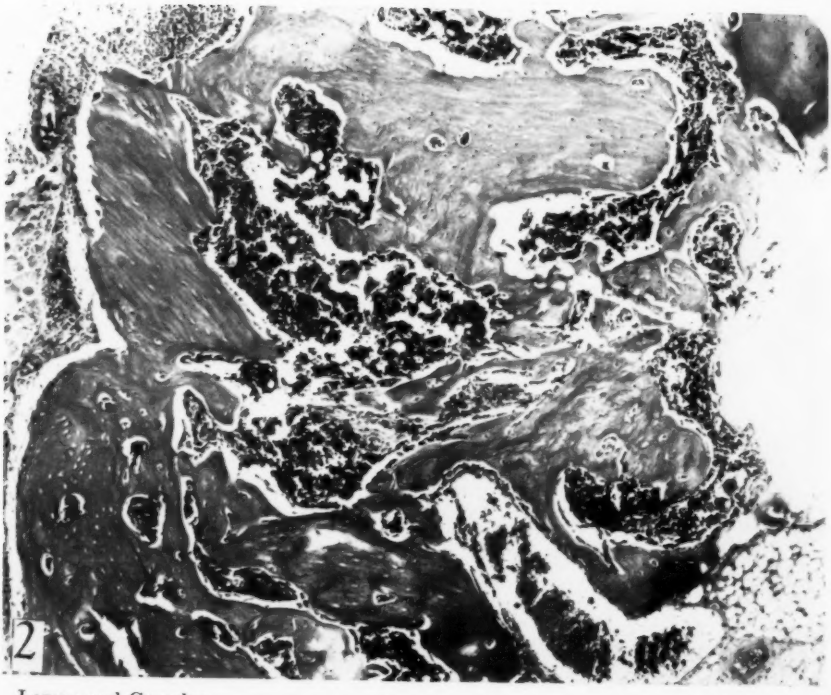
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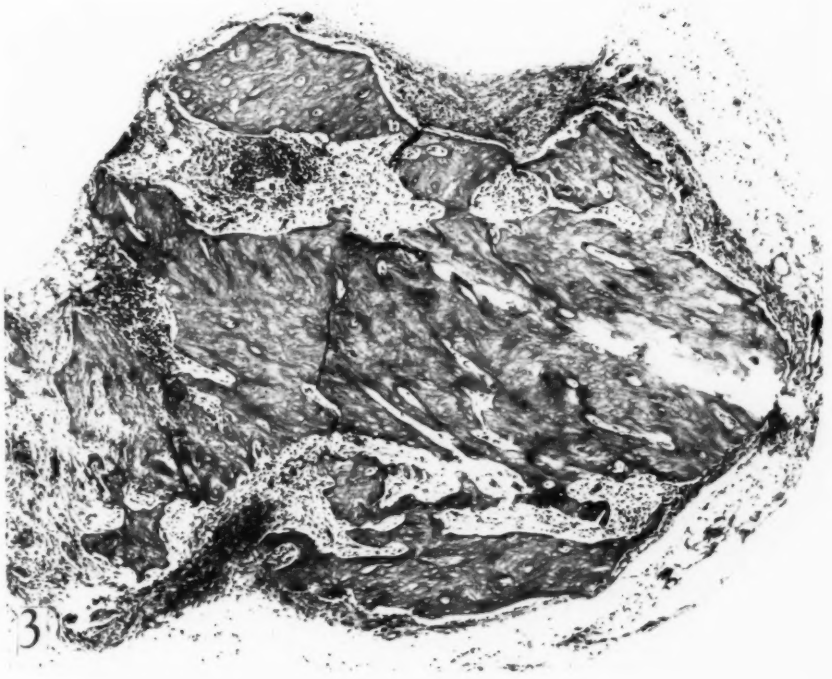
PLATE 80

FIGURE 3.—Section of rat bone recovered from the peritoneal cavity of a mouse about 50 days following 800 r total-body irradiation. Most of the bone is necrotic and the connective-tissue reaction of the host shows many inflammatory cells. No evidence of bone-marrow formation. Hematoxylin and eosin. × 60

FIGURE 4.—Encapsulated mass of ground rat bone recovered from the peritoneal cavity of a mouse 54 days after 800 r total-body irradiation. A few fragments of bone can be recognized in the debris. No evidence of blood formation. Hematoxylin and eosin. × 340



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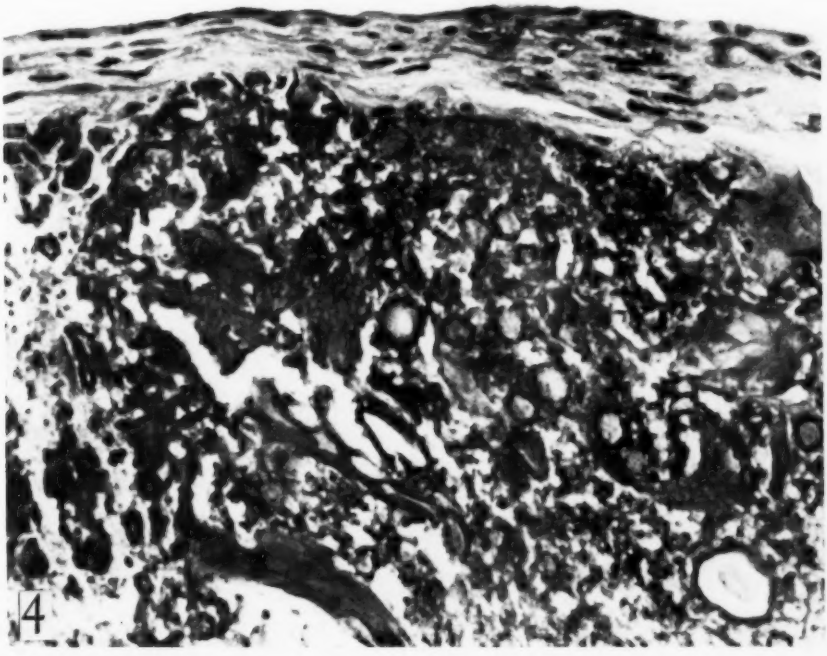

4

The Biological Effects and the Chemical Composition of Podophyllin. A Review[1]

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Part 1. The Biological Effects of Podophyllin (*Margaret G. Kelly*)

For more than 200 years, references to podophyllum, podophyllin, and related substances have appeared in the scientific literature. Although these materials were popular about a century ago as medicinals, chiefly as cathartics and cholagogues, they have been replaced in modern medicine by less drastic purgatives and more effective cholagogues. In 1942, however, interest in these drugs was revived by the report of Kaplan that topical application of podophyllin cured condyloma acuminatum. Since then, a considerable number of papers have been published in many parts of the world on the cytological, biochemical, and pharmacological actions of podophyllin. Clinical investigations are being made of its usefulness for diseases of the skin, particularly verrucae and inflammatory lesions, and in the treatment of benign and malignant tumors. During this period, moreover, the composition of podophyllin has been reexamined, and structural studies of the isolation products have been made. Three new crystalline components (α-peltatin, β-peltatin, and 4′-demethylpodophyllotoxin) have been isolated, and have been found to damage experimental tumors and to cause toxic symptoms in the host similar to those produced by podophyllin and podophyllotoxin. This review was undertaken, therefore, not only to clarify and integrate the more recent knowledge of the biological actions and chemical composition of podophyllin with the older work, but also because it seemed timely to review the experimental and clinical studies to date. It was hoped that these experiences, obtained largely with crude podophyllin, might furnish a basis for future studies with the new crystalline isolation products which now are available.

A. Early Medicinal Uses

Podophyllum peltatum Linnaeus (family: Berberidaceae; common names: May apple, mandrake, Indian apple, wild lemon, duck's foot) is an indigenous, North American, herbaceous perennial flowering in May and bearing fruit in late summer or fall (*156, 266, 247*). Because one of the common names for Podophyllum is mandrake, it is sometimes confused with mandragora (family: Solanaceae).

Records of the use of the roots of *Podophyllum peltatum* as a purgative and emetic extend back several hundred years. Catesby (*54*) in his *Natural History of the Carolinas*, published in 1731, described the May apple and, indeed, included a color plate of the plant in blossom. He noted that the root is "said to be an excellent emetic and

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[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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is used as such in the Carolinas." Catesby listed two botanical references to the plant under the name "Anapodophyllon" (*233, 349*). Champlain (*56*) described the plant and spoke of the fruit as edible, but was unaware, apparently, of any medicinal value of the roots. Jacques Cartier (*52*), however, knew of the root both as a mortal poison and as an antidote for snake venom when applied topically to the bite. According to Brunet (*42*) and Fenton (*112, 113*), the root was considered both a medicine and a poison by the North American Indians; in fact it was used as a suicide agent among the Iroquois. John Uri Lloyd (*208, 209*) stated that podophyllum was used by the Cherokees for deafness and as an anthelmintic, and by the Wyandottes and Southern Indians as a cathartic. Lloyd referred, also, to its use by the Indian doctor, Peter Smith, as an escharotic and in early veterinary medicine.[4]

Bentley (*30*), in 1861, suggested that podophyllin, which he described as a vesicant and rubefacient, might have use as a counterirritant in neuralgic and rheumatic conditions. He noted, in an "American work" loaned to him by M. Bourdas, that it was effective topically for fungus growths, and even for cancerous growths when combined with zinc sulfate and hydrastine.

The early colonists learned of the medical properties of the root from the Indians (*22, 284, 184, 30, 100, 29, 208, 209, 326, 112*). Podophyllum was so firmly entrenched as a part of the *materia medica* of this country that it was included as a cathartic and cholagogue in the first United States Pharmacopoeia, published in 1820. It was listed until the twelfth revision, published in 1942, from which it was dropped. Podophyllum is still included in the British Pharmacopoeia and is, or was, listed in most European pharmacopoeias (German, French, Norwegian, Spanish, Russian).

The resin, podophyllin, was first separated from podophyllum by Dr. John King in 1835 (*182–185*), and not by Mr. William Hodgson as has been stated sometimes (*204, 100, 104, 354*). Dr. King was also the first to administer podophyllin to a patient, and the ill effects of the initial clinical use of the drug are vividly described by him in a letter to Dr. Lloyd (*207*):

> " . . . Having no idea of the intense activity of the article just discovered, I administered about twelve to fifteen grains. Nothing further was thought of the matter until about an hour afterward, when my attention was called to her condition. She was in severe pain and distress, cramps in the stomach and extremities, pulse small and feeble, extremities cold, excessive vomiting and hypercatharsis, and apparently sinking rapidly. Her condition greatly resembled that of a person suffering from a fatal attack of Asiatic cholera. To say that I was greatly alarmed would but feebly describe my mental condition. I ran to secure the aid of two or three professional friends, but could find none of them in their offices. Then I ran back again, trembling over what might be the consequences, and thinking out a course of treatment to pursue. A princely fortune could not induce me to undergo a repetition of such a condition. . . . By perseverance in this course [of treatment], the patient recovered in six or seven days, but unfortunately, with some chronic gastroenteritic abnormal condition, that remained for many years. From this experience I was so influenced that I feared to use any of the remainder of the resin until at least eighteen months had passed, when I ventured a repetition of its use, but in much smaller quantity, and with most excellent results."

With the preparation of podophyllin on a commercial scale in 1850 by Mr. Merrell (*235*) the use of the resin supplanted that of the crude podophyllum. Stories of the new American drug spread to England and the continent of Europe (*30*). Podophyllin did not become official, however, until the fourth revision of the United States Pharmacopoeia in 1863, and, like podophyllum, it was dropped from the twelfth revision in 1942.

Another species of Podophyllum, *Podophyllum emodi* Wallich, grows over most of the Himalayan Range (*89, 65*), and was included in Royle's *Illustrated Botany of the*

[4] We have been unable to find these statements in the reference given, *The Indian Doctor's Dispensatory* by Peter Smith. Browne and Looker, 1812.

Himalayas, published in 1839 (*297*). The natives of this region knew that an aqueous extract of the roots of *Podophyllum emodi* was cathartic. The similarity of the resins of *emodi* and *peltatum* was not recognized, however, until Umney (*351*) published his comparison in 1892, although previously Dymock and Hooper (*103*) had noted the similarity in appearance of the roots of the two species, and had extracted from *P. emodi* a resin with cathartic action. Chopra (*65*) stated that most of the podophyllum used in India was imported from the United States, because the Indians had never attempted to standardize their product and the resin content of *P. emodi* varied with the time of collection and the altitude at which it was grown.

Podophyllin appears to have gained popularity first among the "eclectic" school of practitioners (*182, 263, 262, 241, 206, 207*) and was sometimes referred to as the "eclectic calomel." It was reported to be a cathartic, purgative, deobstruent, vermifuge, hydragogue, cholagogue, choleretic, and expectorant. It was recommended, either alone or in combination with other herbs, for diseases of the liver and kidneys, for scrofula, syphilis, gonorrhea, obstructed menstruation, urinary obstruction, dropsy, and coughs.

The leaves of *Podophyllum peltatum* also were said to yield a resin which had mild cathartic action (*99, 157, 51*). The fruit was considered harmless and edible when ripe, and was occasionally used in preserves (*284, 247, 113*), although a case of poisoning from a brew made with two May apples has been recorded by Reynolds (*290*). The aqueous extract of podophyllin also purged effectively, with griping, when given in high dosage (*281*).

With the increased production of podophyllin, reports of its toxic properties appeared in the literature. Workers in pharmaceutical houses and pharmacists' assistants occasionally developed, on direct contact, pain in the eyes, hyperemia of the iris, cornea, mucous membranes, and eyelids, and rash and eruptions of the scrotum (*322, 158, 365, 368, 246, 64, 256*). Oral and parenteral administration of podophyllin has been followed by serious and even fatal results (*182, 241, 98, 210, 247*).

B. Pharmacological Actions

1. *Effect on intestine and gall bladder.*—Since 1880, when several components were isolated from podophyllin by Podwyssotzki (*273–275*), these and other fractions subsequently isolated have been studied for their physiological and pharmacological effects. In the past, this was restricted chiefly to their ability to produce catharsis or to act as cholagogues and choleretics. This remains a critical action. In the few attempts which have been made recently to treat cancer patients by parenteral or oral route, the limiting factor has been the severe gastrointestinal discomfort which follows the use of high doses of either podophyllin, podophyllotoxin, or the peltatins. Using purgation as the yardstick, podophyllotoxin was generally considered to be the "active" constituent of podophyllin (*223, 93, 92, 321, 258, 354, 192, 352, 39*). The new crystalline isolation products, α-peltatin, β-peltatin, and 4′-demethylpodophyllotoxin, also have been found to produce catharsis (*138, 202*). The other components of podophyllin have little or no cathartic action, although podophylloresin has been described as "active" (*30, 100, 223, 93, 92*). It is of historical interest that Podwyssotzki believed the true active ingredient of podophyllin to be crystalline picropodophyllin. He recognized podophyllotoxin as a physiologically active material, but thought it was formed by the solution of picropodophyllin in podophyllic acid.

MacKenzie and Dixon (*223*) found that resin of *P. emodi* was a more active cathartic than that of *P. peltatum*.

The purgation produced by podophyllin or podophyllotoxin is described as griping and unpleasant, but certain, and it is sometimes accompanied by nausea and vomiting. In spite of this, podophyllin continues to be incorporated into many proprietary medicines and cathartic pills, generally at a level of about 10 mg. per dose (*94*, *289*, *242*). Although it has not been possible to learn the precise quantity of either podophyllum or podophyllin sold annually in the United States today, it is estimated, roughly, that it amounts to carloads.[5]

Some studies have been made to determine the mechanism by which podophyllin and related compounds produce catharsis. Boyd (*38*), using isolated gut preparations, found that podophyllin produced an immediate depression in the rate and amplitude of the rhythmic contractions of the gut, followed by an increase in the force of contraction and a slight loss of tonicity. However, Kelly *et al.* (*178*) reported that no demonstrable change in the contractions of isolated gut was produced by either podophyllotoxin or picropodophyllin, although both compounds provoked diarrhea when given to the intact animal. Dixon and Comb (*93*) administered podophyllin and podophyllotoxin intravenously to cats, and observed increased peristalsis and tone of the intestine after both drugs. This appeared to be a peripheral effect, as it occurred after section of the vagus and splanchnic nerves. In dogs with Thiry-Vella loops, intravenous injection of podophyllin (*141*), or of podophyllotoxin or picropodophyllin (*180*), was followed by loss of tone of the gut anddis appearance of rhythmic contractions. Atropine temporarily blocked this response (*180*). When podophyllin dissolved in alcohol was injected directly into the lumen of the gut loop, there was a temporary relaxation of the gut, followed by an increase in general tonus, marked peristaltic activity, and bluish-black discoloration of the exposed gut surface (*141*).

A distended gall bladder has been noted frequently after administration of podophyllin (*322*, *273–275*, *253*, *354*, *317*, *178*). Podophyllum, podophyllin, and podophyllotoxin have all been described as cholagogues and choleretics, in the United States Pharmacopoeia, yet there is little agreement on this point among investigators. Bain (*17*) reported an increase in the total solids of the bile of the human subject with a bile fistula after nonpurgative doses of podophylloresin, and inhibition of bile secretion after cathartic levels of podophyllotoxin. Bauer and Spiegel (*23*) obtained an increase in the bilirubin content of the blood of patients given podophyllin, and concluded that podophyllin raised the bilirubin level by stimulating the secretion of bile. Podophyllin has been reported also to increase the volume, specific gravity, and the bilirubin and cholic acid content of bile of dogs (*267*). Hazelton (*154*) found that intravenous administration of podophyllin increased the flow of hepatic bile in dogs, but that podophyllin given by intestinal tube caused no change. A

[5] Estimate kindly furnished by S. B. Penick and Co., New York, handlers of crude medicinal drugs.

critical blood-pressure level of approximately 60 mm. of mercury seemed to be necessary for maintenance of biliary flow.

However, Ivy and his co-workers (*162–165*) concluded that neither podophyllum, podophyllin, podophyllotoxin, nor Carter's Little Liver Pills [6] had any detectable effect on the formation of bile, the evacuation of the gall bladder, or the passage of bile into the duodenum, whether administered intravenously, orally, or duodenally to dogs or humans. Other investigators, also, have reported they could find no evidence that podophyllin had any cholagogic or choleretic actions (*18, 272, 324, 53*).

2. *Cardiovascular effect.*—The cardiovascular effects of podophyllin, podophyllotoxin, and picropodophyllin at nonlethal dose levels have been reported by most investigators to be relatively mild, transitory, and irregular in occurrence (*253, 38, 141, 154, 269, 174*). In isolated heart preparations, a depression in rate has been obtained with high concentrations of the drugs (*38, 174, 178*). Blood-pressure measurements in cats and dogs given nonlethal doses showed a transitory rise of a few millimeters of mercury in some animals during injection, or within the next minute or two. An occasional dog responded with a transitory fall of 1 to 3 mm. of mercury, followed by a small rise. Generally, the blood pressure returned to normal within five minutes, with no change in heart rate. In a few instances, extrasystoles and bradycardia have been noted (*269, 174*). At lethal doses, a failing respiration and muscular dystrophy were accompanied by a fall in blood pressure to shock levels (*38, 141, 269, 174*). The blood-pressure changes could be minimized but not completely eliminated by section of the vagus nerves (*141*) or administration of atropine (*269*). Kelly (*174*) made electrocardiographic records of cats and dogs given sublethal and lethal doses of podophyllotoxin. At sublethal levels, the changes in the electrocardiograms were delayed, moderate, reversible, and appeared to be secondary to respiratory difficulties. After lethal doses of the drug, the electrocardiograms showed evidence of myocardial damage: ectopic beats, tachycardia of supraventricular and ventricular origin, irregularities of the S-T segment, disappearance of the P wave, bizarre configuration of the QRS interval, bradycardia, depression of ventricles, and death in cardiac arrest.

3. *Local irritant action.*—Formation of ulcers and sterile abcesses after subcutaneous injection of podophyllotoxin and picropodophyllin have been remarked by several investigators (*274, 223, 93, 92, 354*). Podwyssotzki (*274*) found that picropodophyllin remained unabsorbed at the site of injection. He suggested that the insolubility of picropodophyllin in body fluids was the explanation for the failure of the drug to produce systemic responses on parenteral administration. Recently, as in the past, there have been reports of injury to the eye (*296, 195*) from accidental contact with podophyllin, and of rather severe skin irritation (*173*).

4. *Effect on hematopoietic system.*—The older literature contains no reference to any action of the podophyllin compounds on the hematopoietic

[6] A proprietary containing podophyllum.

system. In the last few years, however, experimental evidence of damage to this system has been obtained, and a few attempts have been made to treat patients having lymphomas and leukemias with these materials. A transitory leukopenia and an eosinopenia have been produced by podophyllin, podophyllotoxin, and the peltatins (*269*, *299*, *300*, *178*, *179*, *177*, *176*, *138*, *214*, *215*, *363*, *364*). Evidence of cytological damage has been observed in the bone marrow, spleen, thymus, lymph nodes (*138*, *214*, *215*, *177*, *176*), and adrenal glands (*177*, *176*). Similar changes have been produced by podophyllotoxin and the peltatins in mice bearing lymphomas and other tumors (*140*), and in normal mice, rats, rabbits, and dogs (*110*). Apparent hyperplasia of reticular tissue and reticular fibers has been noted in the spleen (*214*) and adrenal glands (*177*) of rats given podophyllotoxin. Kelly *et al.* (*176*) concluded from experiments on intact and adrenalectomized rats that a toxic dose of podophyllotoxin exerted a direct cytotoxic action on the hematopoietic and thymicolymphatic systems. A relatively nontoxic dose of the drug, however, gave evidence of both a specific cytotoxic action and a nonspecific adrenotropic action.

5. *Miscellaneous toxic effects.*—Among the other toxic symptoms of of these drugs are: damage to liver and kidneys, hind-leg ataxia in animals, prostration and death (*224*, *241*, *253*, *359*, *91*, *354*, *367*, *332*, *364*). In chickens, but not in mice, MacCardle and Perrault (*220*, *221*) reported damage to the cells of Purkinje, loss of righting reflex, and lack of coordination. Interruption of pregnancy was produced in mice, rats, and rabbits by intravenous or subcutaneous administration of nonlethal doses of podophyllotoxin. Sodium podophyllate, picropodophyllin, and acetyl podophyllotoxin had no effect on pregnancy (*90*).

6. *Metabolism of drug.*—The metabolism of podophyllotoxin was investigated by Kelly *et al.* (*175*). A bioassay method, based on the lethality of podophyllotoxin to the chick embryo, was used in this study. Less than 50 percent of the podophyllotoxin administered to rats and mice could be recovered after one hour by acetone extraction of homogenized whole carcass and excreta. At the end of four hours, less than 10 percent was recovered. Incubation of the drug with tissue homogenates indicated a much slower rate of destruction of podophyllotoxin *in vitro* than *in vivo*. Inactivation of enzyme systems of tissue by heating minimized the loss of podophyllotoxin *in vitro*. There was no evidence from *in vitro* studies with isolated tissues that podophyllotoxin was metabolized preferentially by liver, spleen, kidney, intestine, muscle, or blood serum.

C. Effect on Enzyme Systems

Waravdekar and Leiter (*362*), Waravdekar *et al.* (*361*), and Leiter *et al.* (*198*) reported a marked drop in cytochrome oxidase activity of homogenates of implants of Sarcoma 37 and other transplantable tumors (Lymphoma 1, Lymphoma 2, Leukemia 1210, mammary adenocarcinoma C3HBA, Melanoma S-91) excised from mice given a single subcutaneous

injection of podophyllotoxin, α-peltatin, or β-peltatin. The drop in cytochrome oxidase activity was most rapid in the tumor tissue during the first 8 hours after injection of the drugs, and was measurable before the appearance of "frank necrosis" in the tumor tissue. The cytochrome oxidase activity of homogenates of liver, spleen, kidney, and testis from these animals was not altered as much as that of tumor tissue, even at supralethal doses. During this same interval, no significant change was observed in the dehydropeptidase or aldolase systems of implants of Sarcoma 37 in mice given the drugs. *In vitro* incubation of homogenates of Sarcoma 37 with α- or β-peltatin had less effect on the cytochrome oxidase activity than did injection of these compounds *in vivo*.

Miller *et al.* (*238*, *239*) studied the effect *in vitro* of podophyllotoxin on a variety of enzyme systems. Respiration of lymph nodes, thymus, kidney, spleen, liver, brain, and testis of rats, of Sarcoma 37 implants in mice, and of chick embryo was inhibited. The degree of inhibition increased with time. In the presence of acetate, glucose, alanine, and glutamate, there was marked inhibition of kidney respiration but succinate and pyruvate oxidations were not affected. Miller *et al.* corroborated the observation of Waravdekar and Leiter (*362*) that pretreatment of mice with podophyllotoxin caused marked reduction of *in vitro* respiration of tumor slices. Previous injection of rats with toxic doses of podophyllotoxin reduced respiration *in vitro* of excised spleen, lymph nodes, and thymus, but not of kidney. This effect was minimized by adrenalectomy (*240*).

D. Cytological Effects

Until the report of Kaplan in 1942 (*171*) that topical application of podophyllin in oil cured condyloma acuminatum, the use of podophyllin for its cytological effect was unknown or at least unrecorded.[7] Since then, however, podophyllin and podophyllotoxin have been found to produce mitotic arrest, and their action in this respect is comparable to that of colchicine and other mitotic poisons.

1. *Plant cells.*—B. J. Sullivan and Wechsler (*327*) reported that the meristematic tissue of root tips of *Allium cepa*, which had been immersed in a saturated aqueous solution of podophyllin, showed inhibition of mitosis in prophase, impairment of metaphase with no orientation of chromosomes, and absence of anaphase. Miller *et al.* (*239*) found that podophyllotoxin had no effect on germination of radish and cucumber seeds or corn kernels. T. D. Sullivan (*337*) observed arrest of mitoses in petunia leaves that had been soaked in podophyllin or in colchicine.

2. *Marine eggs, planarians, and newts.*—Cornman studied the effect of podophyllin, podophyllotoxin, and related substances on the maturation and cleavage of eggs of *Arbacia* (*75*) and of *Asterias* (*76*, *77*). Later, Cornman and Cornman (*78*) extended these investigations to include the effect of colchicine and some of the podophyllin compounds on a wider variety of marine eggs. There was "destruction or crippling of the achro-

[7] M. A. Sullivan and L. S. King (*335*) state in a footnote that they were told by Kittredge that urologists in New Orleans had used podophyllin for many years in the treatment of venereal warts.

matic meiotic and mitotic figure," which, if it occurred early in mitosis, prevented cytokinesis. No essential differences in cytological effect could be detected between colchicine, podophyllin, podophyllotoxin, picropodophyllin, or podophyllic acid, but as much as tenfold variation in species susceptibility was noted. Podophyllotoxin was the most active of the podophyllin compounds which were tested; α- and β-peltatin, however, were not included in the study. Quercetin was the only one of the substances that did not block any stage of development, although large doses greatly retarded division. Regeneration in planarians was altered by podophyllin (*131*). Primary healing of the cut surface and subsequent regeneration were retarded, and atypical forms developed. Sentein (*308*), using eggs of *Triturus helveticus*, also compared the effects of the two drugs, podophyllin and colchicine, and found many similarities. Both drugs caused polyploidy if used before fission started; if segmentation had begun, new fissures stopped and blastomeres with polynuclei were formed; and either drug caused depolarization of the spindle.

3. *Microorganisms, protozoa, and worm parasites.*—The colonial character and structure of *Eberthella typhosus* were unaffected by addition of podophyllin to the nutrient broth (*135*). Reiss and Winston (*288*) reported that podophyllin had a fungistatic effect on several species of *Microsporon* and *Trichophyton*. Young (*376*) obtained retardation of growth of *Microsporon audouini* cultures in Sabouraud's media to which podophyllin had been added. Monash (*243*), however, was unable to corroborate the *in vitro* fungicidal activity of podophyllin. Robbins *et al.* (*292*) reported that podophyllotoxin had no effect on infections of *Rickettsia typhi* in the chick embryo.

Diatoms and protozoa moved actively in concentrations of podophyllin that poisoned marine eggs (*78*). The drug had a slightly inhibitory effect on cultures of amoeba (*342*).

Experimental schistosomiasis was not influenced by podophyllin (*304, 305*). Podophyllotoxin, acetylpodophyllotoxin, and α-peltatin were essentially ineffective, also, and showed only questionable activity at toxic dose levels (*217*). Podophyllin has been described as an effective vermifuge, first stimulating, then paralyzing, and finally killing *Ascaris* (*258*).

4. *Condyloma acuminatum and normal epithelial tissue.*—In 1945, Marchi (*228*) investigated the effect of podophyllin and of colchicine on condyloma. He noted vasodilation, edema, mitotic arrest, pyknosis, and destruction and sloughing of tissues after topical application of either of these drugs. The same year, Bourg and Dustin (*37*) reported cures of papilloma vulvaris by topical application of colchicine. In 1946, King and Sullivan (*189*), working independently, described pronounced cytological changes in normal human and rabbit skin, and in condyloma acuminatum following local treatment with either podophyllin or colchicine. The cytological effects produced in normal epithelium were comparable to those observed in condyloma. The histological and clinical effects observed in condyloma acuminatum after application of colchi-

cine more intense and of shorter duration than those obtained following podophyllin application. Radaeli (*282*, *283*) also compared the effect of podophyllin and colchicine on condylomas. In a later series of papers, King (*186*, *187*), and King and Cauldwell (*188*) made precise cytological observations of the effect of a single application of podophyllin on mouse skin.

King and Sullivan (*190*) described the cytological effects of podophyllin as follows:

> "The most characteristic change is the production of enlarged, swollen cells with finely reticulated, palely basophilic cytoplasm and dispersed chromatin material which shows some type of chromosomal arrangement. Other changes, more frequently seen, such as eosinophilic cells with pyknotic nuclei, various forms of cytoplasmic vacuolization and nuclear alterations, are described in detail but are not claimed to be specific."

The histological findings of King and Sullivan have been confirmed by many investigators (*34*, *74*, *44*, *45*, *114*, *280*, *371*, *373*, *356*, *264*). In addition, Vilanova *et al.* (*356*) called attention to formation of vacuolar cavities in cells of the Malpighian layer; Buccellato (*45*) described changes in "Lipschütz cells" of condyloma, and vascular damage to capillaries; and Ferrari (*114*) emphasized the inflammatory phenomena apparent in derma and epidermis. Ballerio (*20*) observed mitotic arrest and cell damage of vesical epithelium in rabbits after application of podophyllin. Regeneration and repair occurred unless the treatment was so severe as to cause scarring. Sullivan and Blanchard (*331*), Sullivan *et al.* (*333*), and Sullivan (*328*) postulated that podophyllotoxin was the component of podophyllin which was responsible for the damaging action on cells. Berenblum (*31*) could demonstrate no carcinogenic or cocarcinogenic action of podophyllotoxin in polyethylene glycol on the skin of mice.

Confirmatory evidence that podophyllin acts as a mitotic poison has been obtained with indirect methods. Isler (*160*, *161*) demonstrated that topical application of podophyllin to the nipple of the guinea pig caused arrested mitoses if the nipples had been stimulated previously by injection of the animal with an estrogen, but was without effect on untreated guinea pigs. The histological picture was similar to the "Dustin effect" produced by parenteral administration of colchicine. Brun *et al.* (*41*) obtained the "Dustin effect" in the thymus, as well as in the guinea pig nipple, by either topical or parenteral administration of podophyllin. Picropodophyllin was inactive. Didcock *et al.* (*90*) found topical application of podophyllotoxin to the vagina of rabbits inhibited vaginal response to systemic administration of estrogen, thus suggesting interference with epithelial growth. In 1944, Culp *et al.* (*83*) made the tentative suggestion that podophyllin acts on condyloma by producing local vascular spasm, ischemia, and sloughing. However, it is generally accepted now that the drug acts directly on epithelial cells, producing mitotic arrest and degeneration. Bizarre cells are formed, which have been called "podophyllin cells" by Sullivan and King (*335*), and are similar to the "colchicine

figures" described by Dustin (*101*), Lits (*205*), Ludford (*216*), and others after parenteral administration of colchicine. Furthermore, it has been suggested that podophyllin causes derangement and disorders of cell metabolism through a direct cytotoxic action (*335*, *114*, *33*, *43*).

Sullivan and King (*335*) studied the effect of single and repeated daily applications of podophyllin to the forearm or prepuce of normal men, and to the skin of rabbits. They concluded that "resin of podophyllum is a cutaneous sensitizer of the first order." Podophyllotoxin had a similar action (*328*). Recently, Sullivan and Hearin (*334*) found that the peltatins caused fewer skin reactions than podophyllin or podophyllotoxin. Other investigators, however, have concluded that podophyllin acts primarily as a skin irritant and not as an allergen (*280*, *74*, *356*, *358*).

5. *Experimental tumors.*—Interest was stimulated in the possible action of podophyllin on tumor tissue by reports of the cytological alterations produced by the drug in normal epithelium and condyloma. Several papers describing the damaging effect of podophyllin on experimental tumors were given at scientific meetings held in the spring and fall of 1947. Belkin (*27*, *28*) reported that repeated subcutaneous injections of podophyllin caused necrosis and retardation of growth in tumor implants. Hartwell and Shear (*153*) produced hemorrhage and necrosis of intramuscular implants of Sarcoma 37 in mice with a single, subcutaneous, sublethal dose of podophyllin, podophyllotoxin, or α-peltatin. Ormsbee and Cornman (*259*, *260*) reported that podophyllin had a toxic effect and repressed growth in tissue cultures of sarcoma L 946A II and lung sarcoma MA387, but had only slight, reversible effects on cultures of mouse embryonic skin. Podophyllotoxin also damaged tumor cells *in vitro*, but acted within a more restricted dose range (*261*). Sarcoma 180, growing on the chorio-allantoic membrane of chick embryo, was not damaged by the maximum tolerated dose of podophyllin, podophyllotoxin, or quercetin (*172*). Immersion of tumor fragments in podophyllin prior to implantation into mice markedly inhibited growth of tumor transplants; little or no inhibition was detected in fragments immersed in podophyllotoxin or quercetin (*325*). MacCardle and Downing (*219*) described cytological evidence of damage (mitotic arrests, distorted chromosomal distribution, multinucleation, necrosis) in Sarcoma 37 and Rous sarcoma after parenteral administration of podophyllin. Mitotic arrest was seen in the intestinal mucosa as well as in tumor tissue. Microscopic evidence of alterations in tumor tissue could be detected within two hours after a single injection of podophyllin. Microincineration of tumor tissue revealed an increase in calcium and magnesium ash, or both, in the achromatic figures (*218*).

Leiter *et al.* (*199*, *200*) reported that podophyllotoxin, α-peltatin, and β-peltatin all produced damage to Sarcoma 37 in mice. More recently, Leiter *et al.* (*202*) have found that a new crystalline isolation product of *P. emodi*, 4′-demethylpodophyllotoxin, also damaged Sarcoma 37. The cytological effects on tumor tissue of these four crystalline isolation products of podophyllin have proved to be similar to those produced by

podophyllin. Massive doses of picropodophyllin, podophyllic acid, quercetin, or picropodophyllin glucoside caused no gross evidence of tumor damage. On microscopic examination, however, distorted mitotic figures were seen in tumors of animals treated with picropodophyllin or quercetin. Using the transparent-chamber technique, Algire *et al.* (*6*, *5*) and Algire (*2–4*) have observed vascular changes (ischemia, capillary stasis, hemorrhage) in Sarcoma 37 implants of animals administered podophyllotoxin. Picropodophyllin had no effect. These changes have been accompanied by fall in blood pressure (indirect measurement) in an artery of adjacent normal tissue. Greenspan *et al.* (*139*, *140*) found that maximum tolerated doses of α- or β-peltatin were required to produce significant cell damage in transplanted adenocarcinoma, carcinoma, or melanoma. In leukemic mice, the peltatins caused leukopenia, shrinkage of lymphoid tissue and lymphoid tumors, but never caused complete regression of the tumor.

A number of materials structurally related to podophyllotoxin were tested by Leiter and Hartwell (*201*) for their effect on Sarcoma 37 implants. The acetyl derivative of podophyllotoxin, and the acetates and methyl and ethyl ethers of the peltatins, damaged the tumor. The acetyl derivative of picropodophyllin, like the parent compound, had no effect. Several benzoic acid derivatives, which were structually similar to products formed by oxidative degradation of podophyllotoxin or the peltatins, were inactive.

Muñoz *et al.* (*248*) inhibited growth of transplants of Roffo sarcoma in rats, and prevented appearance of metastases by administration of podophyllin. Waterman (*363*, *364*) obtained inhibition of growth of implants of spontaneous mouse carcinoma and lymphosarcoma, and of benzpyrene-induced sarcoma by repeated injections of podophyllotoxin. Loran and Guth (*211*) and Loran and Towbin (*212*, *213*)described "lysis" of Sarcoma 37 implants in Swiss mice injected either with podophyllin or with a "new" material which they isolated from podophyllin but did not characterize chemically. Makino and Tanaka (*226*, *227*) used an ascites tumor, the Yoshida rat sarcoma, to study the action of podophyllin. Within one hour after intraperitoneal injection of the drug, distorted mitotic figures, mitotic arrest, and disintegration were seen. The smaller-sized tumor cells seemed less affected by the drug, and within two or three days were dividing again by normal mitosis. About fourfold increase in survival was obtained in animals given three treatments.

E. Recent Clinical Uses

Kaplan's paper in 1942 (*171*), reporting cure of condyloma acuminatum by topical application of podophyllin, aroused the interest of clinicians. Since then, podophyllin has been tried in the treatment of a number of diseases, particularly those characterized by warty lesions of the skin, and neoplasms occurring in regions of the body accessible to topical therapy.

1. *Diseases of the skin due to infectious agents (verrucae, granuloma inguinale and other granulamatous lesions, molluscum contagiosum, tinea capitis).*—Kaplan (*171*), Culp and Kaplan (*82*), and Culp *et al.* (*83*) reported cures in a large series of cases of condyloma acuminatum treated with podophyllin suspended in mineral oil or incorporated into an aqueous paste. There was no immediate reaction, but in a few hours the growths appeared blanched and the patient felt pain. In 12 hours, the local reaction became marked and was characterized by inflammation and edema. In 24 to 48 hours the treated areas appeared necrotic, and in two or three days the growths started to slough off. Most lesions disappeared within four days after a single application, although growths within the urethra required more treatments. There was no ulceration or scarring, and convalescence was generally painless, although sedation was necessary in some patients with extensive lesions. No systemic reactions were experienced.

After the initial reports by Kaplan and his associates in 1942 and 1944, the effectiveness of podophyllin therapy for condylomas was confirmed in this country and elsewhere. In 1945, successful treatment of condyloma acuminatum with podophyllin was reported by: Gersh (*130*) and Sulzberger and Baer (*338*) in the United States; Haber (*145*), MacGregor (*222*), and Csonka (*81*) in England; Touraine (*346*) in France; Emanuel (*107*) in Denmark; Marchi (*228*) in Italy; Beheran and Kraves (*25*) in Argentina; and Serrano Camargo (*309*) in Colombia. In the next few years, satisfactory clinical results with podophyllin therapy for condyloma were obtained by Alvarez and Zardini Schmiot (*7*) in Brazil, Borelli (*34*) and Wyss-Chodat (*371*) in Switzerland, Trolle (*350*) in Sweden, Sainz de Aja (*298*) and Apalategui Asúa (*12*) in Spain, Pierard and Legros (*271*) in Belgium,[8] Simons (*311*) in Holland, and many others (*8, 32, 50, 57, 67, 72, 159, 189, 236, 244, 1, 14, 44, 87, 115, 117, 128, 129, 132, 229, 231, 270, 279, 291, 295, 203, 245, 254, 255,* and others). Podophyllin is now considered the treatment of choice for condyloma acuminatum because of its effectiveness, the simplicity of treatment, and the absence of severe pain to the patient. Podophyllotoxin and the peltatins, also, have been used successfully to treat condylomas (*333, 328, 334*). The peltatins have the added advantage of producing only negligible skin reactions (*334*). Podophylloresin, quercetin, picropodophyllin, or podophyllic acid have no effect (*335*).

Modifications in treatment have been made by various clinicians (*1, 8, 50, 67, 72, 88, 118, 128, 264, 370*). Podophyllin has been applied in oil, in vaseline, in 95 percent alcohol, in dextran, as a paste, in powder form, or in various other vehicles and dosage forms. It can be left on from 2 to 8 hours, and may be repeated daily, weekly, or at longer intervals. The reported incidence of untoward reactions differs among investigators, but there seems to be general agreement that rather severe reactions may occur among uncircumcised men treated in the prepuce area. Whatever

[8] An earlier paper by these authors in *La Scalpel*, 98: No. 9, 156, 1945, is sometimes cited, but the reference is inaccurate and could not be located in *La Scalpel*, 98: No. 9.

the site, if the lesions are extensive it is better to treat in smaller areas rather than to apply the drug over the entire surface at one time. All patients are cautioned against getting the drug in the eyes, and care is recommended to avoid unnecessary spread of the medication to adjacent normal tissue (*296*, *173*, *195*, *291*, *108*, *120*).

The consensus seems to be that podophyllin is not effective against verruca vulgaris (*82*, *57*, *237*, *10*, *12*, *73*, *121*, *144*, *190*, *271*, *279*, *335*, *358*, *15*, *347*, and others), presumably either because it is unable to penetrate hyperkeratinized areas, or, as Vilanova (*358*) suggests, because the tissue is not actively proliferating. With verruca plana, podophyllin has been reported by some investigators to produce cures (*279*, *358*, *71*, *168*, *170*), and by others to have no effect (*12*, *144*, *347*, and others). Podophyllin has been used as a treatment for plantar warts (*10*, *194*, *373*, *71*, *13*, *24*, *26*, *102*, *360*). It is successful only when the warts are pared down before applying the drug, and the surface bound tightly afterward. Several applications are necessary. Sullivan and Hearin (*334*) have pointed out that the peltatins may prove effective against horny verrucae because they can be applied in alkali solution without appreciable loss of potency. Persistent buccal warts on a racing greyhound were cured in 36 hours after podophyllin had been injected into the submucosa (*191*).

Tomskey *et al.* (*345*) treated granuloma inguinale with 20 percent podophyllin in olive oil. All the cases improved, and the response was far superior to that evoked by any other treatment they had used. These results have been confirmed by Smith (*313*). Culp and Kaplan (*82*) found podophyllin capable of destroying most types of granulomatous tissue. Conejo Mir (*69*, *70*) reported cure of granuloma pyogenicum of the upper lip with podophyllin in alcohol, and Cordero (*72*) markedly reduced the size of botryomycoma of the toe with 4 applications of 25-percent podophyllin in liquid vaseline. Foix (*120*) used 25-percent podophyllin in mineral oil to treat two patients with granuloma which occurred after hysterectomy in vaginal scar tissue. The results were satisfactory, the method was simple, the patients experienced a minimum of discomfort, and there were no recurrences within one year.

Hall (*146*), Barefoot (*21*), and Craps (*80*) have reported that application of podophyllin dried up lesions of molluscum contagiosum. Tanissa (*340*), however, used podophyllin therapy in one case of molluscum contagiosum with no effect.

The possible usefulness of podophyllin in treatment of fungus infections of the skin was postulated by Michel (*237*) and Chatain (*57*) in 1946, because of the similarity in some of the local effects of podophyllin and chrysarobin, an antifungus agent. Since then, podophyllin has been tried in tinea capitis with equivocal results. Reiss and Doherty (*286*, *287*), and Young (*376*) reported beneficial results in about 30 percent of patients treated (Reiss and Doherty, 105 cases; Young, 14 cases), while Schwebel *et al.* (*306*) found the drug ineffective in a series of 31 children.

2. *Nonspecific dermatoses* (*eczema, psoriasis, neurodermatoses, other dermatoses*).—Recently, podophyllin has been tried in the treatment of

allergic and inflammatory conditions of the skin. Symptomatic relief has been obtained in some of these diseases, particularly those characterized by moist lesions or pruritus.

In 1947, Sullivan and King (*335*) noted an apparent "drying effect" of podophyllin on normal skin and mucous membranes, and described microscopic alterations suggestive of this action in an area of eczematous dermatitis to which the drug had been applied. Vilanova and Cardenal (*355*), and Vilanova *et al.* (*356*, *357*) also observed a "drying effect" of podophyllin and podophyllotoxin on epidermis, and made the first clinical application of these observations to treatment of exudative contact dermatitis and eczema. The lesions dried and the itching was lessened. Confirmatory results have been obtained by Conejo Mir (*69*, *70*), Ferrari (*114*), Chubarova (*66*), O'Farrell (*257*), Parant (*262*), and Nelson (*252*).

Michel (*236*, *237*) compared the action of podophyllin and chrysarobin on lesions of psoriasis, and concluded the effects (reddening, sloughing, etc.) of the two drugs were analogous. Prassoli and Boveri (*280*) obtained clinical remissions of variable duration in 15 cases of psoriasis treated with repeated applications of 25-percent podophyllin in alcohol-glycerin. Histologic studies showed nuclear and cytoplasmic changes within 24 hours after the drug was used. Touraine (*347*), Dainow (*85*), Ferrari (*114*), Garb (*125*), Nelson (*252*), and Smith (*316*) also have found the drug effective in psoriasis. Danda (*86*), however, obtained only slight symptomatic improvement with 2-percent podophyllin in alcohol-glycerin.

Garb (*124*, *126*) treated circumscribed neurodermatitis (lichen simplex chronicus) with podophyllin. In 5 of 7 cases that had resisted X-ray therapy, there was complete regression of lesions, pruritus was allayed in all patients, and there were no recurrences within 2 years. These results have been confirmed by O'Farrell (*257*), who treated successfully 18 of 20 patients with hypertrophic lesions of neurodermatitis, and by Nelson (*252*). Equivocal results were obtained in one case of chronic radiodermatitis treated with podophyllin (*11*).

3. *Metabolic diseases* (*amyloidosis cutis*, *acanthosis nigricans*, *gout*, *rheumatoid arthritis*).—The use of podophyllin in dermatoses of metabolic origin gives some indication that it may be of value in these diseases, but as yet the results are too scanty to evaluate the efficacy of treatment. Garb (*123*, *125*) treated an X-ray-resistant case of amyloidosis cutis of 15 years' duration with podophyllin. The itching was stopped, and although hyperpigmented areas persisted, they became smaller. Epstein (*109*) used podophyllin in a case of acanthosis nigricans with some success. Sullivan (*330*) found parenteral administration of podophyllin ineffective in gout. Recently, Cervini and Pagni (*55*) reported completely negative results in four cases of rheumatoid arthritis treated with podophyllin, and no evidence (eosinopenia) of adrenal cortical stimulation. The drug was given orally, 5 mg. per dose, 3 times daily, for 7 days.

4. *Benign new growths.*—Topical application of podophyllin has been effective in the treatment of some types of benign new growths. With these lesions, as in condyloma acuminatum, podophyllin therapy has ad-

vantages over surgery, electrocautery, or X rays. It is effective, relatively painless, and causes a minimum of discomfort to the patient; it is simple and inexpensive, can be used repeatedly if necessary, and gives good cosmetic results.

Culp and Kaplan (*82*) found podophyllin ineffective against benign rectal polypi, but Farnarier (*111*), reported cures in two cases of multiple urogenital polypi. In 1946, Cordero (*72*) treated successfully a voluminous papilloma of the nasal fossa with six applications of podophyllin and curettage. The following year, Reich *et al.* (*285*) treated eight cases of soft papilloma of the female urethra with podophyllin. In 2 to 5 days, the papillomas shriveled and sloughed off in much the same way that condylomas do. Hollingsworth *et al.* (*155*) used podophyllin in alcohol in treating five cases of juvenile papilloma of the larynx. The drug was applied either daily for 4 days or weekly for 8 treatments. No systemic or local reactions were experienced. The papilloma disappeared in three cases, and was reduced in size in the other two. Bucalossi and Di Pietro (*43*) used podophyllin on a papilloma of the penis with beneficial effects. A recurrence responded to a second course of treatment with podophyllin, and no further recurrences were experienced. In a patient with multiple, benign, cystic epithelioma, reported by Zell and Sullivan (*377*), podophyllin had no effect.

In 1948, Semple (*307*) treated four cases of papilloma of the bladder with podophyllin in liquid paraffin. The drug was applied through a cystoscope directly to the lesion. The papillomas decreased to a size that could be treated successfully by periurethral fulguration. Comparable results were obtained with this method of application by Duckworth (*97*) and Andrade (*9*). Van Keerbergen (*353*) administered podophyllin through a catheter to avoid contact with the urethra. He found this method of treatment satisfactory in cases of widely disseminated, small papillomas of the bladder, but preferred electrocoagulation or resection for large growths. In 1948, Gonzales and Firstater (*133*) also obtained encouraging results in a few cases of vesical papilloma treated either by insufflating powdered podophyllin into the bladder, or instilling a suspension in mineral oil. Later, Firstater (*116*) reported cures in five cases of disseminated vesical papilloma treated with podophyllin. He instilled increasing concentrations of podophyllin (from 3 to 30 percent) as a suspension in mineral oil, at intervals of 4 to 7 days. Jarrams (*166*), however, observed no apparent effect on growth of a papilloma of the bladder which he treated by repeated instillations of podophyllin. In this patient, two calculi composed of podophyllin were formed.

Senile keratoses have responded satisfactorily to topical application of podophyllin (*279, 146, 134, 316, 252*).

5. *Malignant new growths.*—Cutaneous carcinoma, both of squamous-cell and basal-cell type, has been treated with podophyllin. The drug is generally applied as a 25-percent suspension in mineral oil, or incorporated into a paste, or dissolved in alcohol. Treatment schedules have varied

from daily application for a few days, to intermittent application over a period of several weeks.

Cures of "superficial epitheliomas" have been reported by Wyss-Chodat (*372–375*), Sullivan (*329*), Hall (*146*), Smith and Garrett (*314, 315*), Ellis (*106*), Ballarati (*19*), Larsson (*197*), Rocchietta (*294*), and Nelson (*252*). The incidence of recurrence of these intradermal lesions is about the same as that experienced with other types of therapy, and further treatment with podophyllin has been effective.

Infiltrating squamous-cell and basal-cell carcinomas also have been treated by topical application of podophyllin. The results have not been satisfactory, although microscopic evidence of damage to deep-lying malignant tissue has been observed in many instances. High incidence of recurrence after initial superficial healing, or, less commonly, no effect of treatment, have been reported by Wyss-Chodat (*371–375*), Sullivan *et al.* (*336*), Touraine (*347*), Garb (*122, 127*), Kern and Fanger (*181*), Larsson (*196, 197*), de Graciansky (*134*), Perdrup (*265*), Smith and Garrett (*315*), Sims and Pensky (*312*), Wolf (*369*), Baer (*16*), Bucalossi and Di Pietro (*43*), and Jørgsholm (*169*).

Recently, Smith (*316*), in summarizing his experiences with podophyllin therapy of skin carcinoma, has expressed what appears to be the current opinion:

> "The method has virtue, and should not be forgotten, though it is not for routine use. Neither is it for haphazard use, and careful attention to the details of technique must be observed. We do use podophyllin almost routinely in our office in the treatment of multiple superficial basal cell epitheliomatosis, a relatively benign and superficial condition. The condition can be handled with the drug with much less scarring than with other methods, and the lesions can be just as surely cured if one is careful to observe the points of technique necessary, such as removal of all necrotic tissue before each application."

Sulzberger (*339*) suggested that pretreatment of epitheliomas with podophyllin or podophyllotoxin might, by arresting mitosis, make the tumors more sensitive to X rays. Ballarati (*19*) treated successfully with X rays basal-cell carcinomas that had failed to respond to podophyllin. He, too, suggested that combined podophyllin and X-ray therapy might prove very useful. Excellent results have been obtained by treatment with podophyllin of recurrences after X-ray therapy of cutaneous carcinoma (*265, 197*), and of carcinoma and precancerous lesions caused by X-ray injury (*252, 315, 11*).

Collet and Vellut (*68*) used podophyllin to treat 12 dogs with Sticker sarcoma, a transmissible, venereal lymphosarcoma which frequently undergoes spontaneous regression. The superficial growth disappeared, and although large, infiltrating tumors did not disappear, they were diminished sharply in size, and thus surgical removal was facilitated.

Bunge (*47*) tried podophyllin in one case of adenocarcinoma of the bladder. The bladder was exstrophic, with epispadias. A 10-percent

suspension of podophyllin in mineral oil was applied daily for 3 weeks over the exposed bladder surface. No gross or microscopic changes were observed either in the normal bladder mucosa or in the neoplasm, which continued to grow.

Ballarati (*19*) applied dressings impregnated with 25-percent podophyllin in alcohol-glycerin to ulcerated lesions associated with advanced carcinoma of the breast. The dressing was removed in 2 to 4 hours, and treatment was repeated twice weekly for periods of 3 months to more than a year. Transitory, palliative effects (decrease in bleeding, decrease in local secretions and crusting, alleviation of pain) were attributed to podophyllin in 2 of 3 patients, but the clinical course of the disease was unaltered.

A few attempts have been made to administer podophyllotoxin or the peltatins by intravenous, intramuscular, or oral routes to patients with advanced neoplasms in parts of the body not accessible to topical treatment. The therapeutic value of systemic injections of the active isolation products of podophyllin cannot be determined until more clinical data becomes available.

Calabrese (*49*) used intramuscular injections of suspensions of podophyllotoxin in olive oil (20 mg. dose) in two patients, and intravenous injections of podophyllotoxin as a colloidal suspension in alcohol-water (5 mg. dose) in a third patient. The drug was given twice weekly, and produced no toxic symptoms except mild intestinal discomfort in one patient. One case, an inoperable bronchial carcinoma with cerebral and cerebellar metastases, showed clinical improvement. The neurological symptoms disappeared almost completely, and clearing of the lungs was demonstrable by roentgen examination. Another patient, with pulmonary metastases from a carcinoma of the uterus, showed clinical and radiological improvement. In the third case, which had an enormous pelvic metastasis following surgical removal of carcinoma of the uterus, the only effect which could be attributed to podophyllotoxin was relief of sciatic pain. All the patients either had been treated with a chromosomal material prepared from sperm cells before podophyllotoxin was given, or were given both medications concurrently. Ballarati (*19*), did not observe any beneficial effect with intravenous injection of podophyllotoxin in the following inoperable cancers: an epithelioma of the larynx, lymphoepithelioma of the tonsil, 3 tumors of the buccal cavity, and 2 ulcerated carcinomas of the breast. The parenteral use of podophyllotoxin was suggested to Ballarati by Wyss-Chodat, who was trying this route of administration on his own patients. The drug was well tolerated; the blood picture was not changed; and no discomfort or toxic symptoms developed after as many as 40 to 50 injections of 20 to 30 cc. of 0.1 percent aqueous suspension of podophyllotoxin.

Greenspan *et al.* (*136, 137*) gave α-peltatin to 45 patients with advanced neoplasms, including 27 malignant lymphomas, 6 adenocarcinomas, 7 squamous-cell carcinomas, 3 anaplastic carcinomas, and 2 malignant melanomas. It was concluded that treatment with α-peltatin did not

alter significantly the clinical course of incurable cancer. The drug was given intravenously as an infusion in glucose solution, generally at a dose level of 0.15 to 0.30 mg. per kg., at intervals of 1 to 3 days. The maximum total dose was 170 mg., administered in 11 injections within 22 days. At toxic dose levels, there was transient reduction in splenomegaly and lymphadenopathy in patients with malignant lymphomas, and microscopic evidence of induced necrosis in some of the patients with ulcerating squamous-cell carcinoma. One patient with giant follicular lymphoblastoma experienced clinical remission of 18 months' duration. Combination therapy of α-peltatin and aminopterin resulted in brief clinical remissions in two patients with acute leukemia. More intensive treatment schedules or higher dose levels of α-peltatin produced severe diarrhea and asthenia, and other toxic reactions such as nausea, vomiting, chills, fever, and pain referred to the neoplastic lesion. Extensive laboratory tests were made during the course of treatment, and no evidence was obtained of damage to liver, kidney, heart, central nervous system, or hematopoietic system.

β-Peltatin, according to unpublished results of Downing *et al.* (*96*), has been ineffective when given by mouth to children with acute or chronic leukemia, neuroblastoma, and other widely disseminated neoplasms. Details of treatment were not available.

Preliminary studies of the effect of podophyllin on mycosis fungoides indicate that topical application of the drug, while not a cure, alleviates the discomfort of the patient, and may have some therapeutic value (*119, 167, 79*).

Summary

Judging from the medical claims which have been made for podophyllin in the last three centuries, the drug could be a panacea. Twice within the last 150 years, reports from the United States of therapeutic properties of podophyllin have attracted attention of clinicians and research workers all over the world.

In the eighteen hundreds, the drug was recognized and used chiefly as a drastic purgative, and as a cholagogue and choleretic. It was administered by mouth or by injection. Direct contact with skin and mucous membranes was avoided because of local irritation, although a few references can be found to its use, topically, as a counterirritant and to destroy "cancerous growths." Today, in antithesis to the old concept, the cytotoxic action of podophyllin forms the basis of scientific interest in the drug and of its therapeutic use in the treatment of condyloma acuminatum. The severe gastrointestinal effects are regarded as toxic symptoms which limit dosage.

The pharmacological actions of podophyllin and podophyllotoxin on the gastrointestinal and cardiovascular systems of man and animals were studied extensively by the older investigators. Recent work has confirmed the earlier findings and has extended them to include three new crystalline isolation products of podophyllin, α-peltatin, β-peltatin, and

4′-demethylpodophyllotoxin. So far as can be judged, the crystalline isolation products of podophyllin produce the same pharmacological effects as the parent crude resin.

Administration of these drugs by oral or parenteral route causes catharsis. The peristaltic activity of the gut is increased, there is gradual loss of tone, and, finally, disappearance of rhythmic contractions. The action appears to be peripheral. The cardiovascular effects of sublethal doses of podophyllin or its active crystalline isolation products are relatively mild and transitory. After lethal doses, fall in blood pressure to shock levels has been reported secondary to a failing respiration, and electrocardiographic evidence of myocardial damage has been obtained. There is little effect on the central nervous system, although hind-leg ataxia has been noted occasionally in dogs, and damage to the cells of Purkinje, loss of righting reflex, and lack of co-ordination have been described in chickens.

New contributions to the pharmacology of the podophyllin compounds have been made within the last decade. A cytotoxic action similar to that of colchicine has been demonstrated. This effect (mitotic arrest, nuclear fragmentation, other evidence of cellular damage) has been observed in skin, intestinal mucosa, and tumor tissue after parenteral administration or topical application of these materials. At relatively nontoxic (no diarrhea, no loss of weight) dose levels, the peripheral blood leukocytes are reduced, the bone marrow appears hypoplastic, spleen and other lymphatic tissue are decreased in size, the adrenal cortex is depleted of lipid, and marked necrosis and hemorrhage is produced in experimental tumors. The respiratory enzymes of lymphatic tissue and of tumor implants in mice are depressed within a few hours after injection of the animal with podophyllin or one of its crystalline isolation products.

The extension of topical use of podophyllin to treatment of benign and malignant new growth followed demonstration of its therapeutic effect on condyloma, and recognition of its cytotoxic action on normal and pathological tissue. There have been a number of reports of successful treatment of papilloma of various sites, of senile keratoses, and of intradermal carcinoma. The drug is not recommended for infiltrating squamous- or basal-cell carcinoma because of the high incidence of recurrence after apparent healing. It has produced only mild palliative effects on ulcerative carcinoma of the breast.

A few attempts have been made to treat inoperable systemic cancer by parenteral or oral administration of podophyllin or one of its components. No evaluation can be made until more data have accumulated. To date, however, no unequivocal therapeutic effect has been obtained. Some microscopic evidence (mitotic arrest, necrosis) of damage to tumor tissue has been described in biopsy and autopsy material, but no effect on the hematopoietic system has been observed.

There has been some indication that podophyllin sensitizes tissue to the action of X rays. The usefulness of such an effect in the management of cancer and many other diseases now treated with X rays is obvious,

and it is hoped that future studies will provide definitive information.

Podophyllotoxin, α-peltatin, β-peltatin, and 4′-demethylpodophyllotoxin have pharmacological properties similar to those of podophyllin, but cause fewer undesirable or unpredictable side-effects, permit precision dosage, and afford a better margin of safety. These crystalline isolation products of podophyllin (except for demethylpodophyllotoxin) have now become available commercially, and their use in future experimental and clinical studies should be of great advantage.

Part II. The Chemistry of Podophyllin (*Jonathan L. Hartwell*)

The dried rhizomes and roots of the plant, *Podophyllum peltatum* L. (fam. Berberidaceae), are known as American podophyllum or podophyllum N. F. The dried rhizomes and roots of the related species, *P. emodi* Wall., constitute Podophyllum Indicum or Indian podophyllum. Both of these podophyllums are recognized by the British Pharmacopoeia[9] (*39*), neither by the U. S. Pharmacopoeia fourteenth revision (*268*), and only the former by the National Formulary (*251*). A third species, *P. sikkimensis*, growing near the Himalayas, has recently been named (*63*) and studied chemically (*62, 61*).[10]

When any of the podophyllums is extracted with alcohol, the concentrated extract precipitated with water acidified with hydrochloric acid (in the N. F. process), and the precipitated solid washed thoroughly and dried at a low temperature, a light brown to greenish-yellow amorphous solid is obtained called variously podophyllum resin N. F., resina podophylli, or podophyllin. Only the resin from *P. peltatum* is recognized by the N. F., while those from both *P. peltatum* and *P. emodi* are official in Great Britain. For the purposes of this review, the former will be called American podophyllin and the resin from *P. emodi* Indian podophyllin. American podophyllum contains 4 to 6 percent of resin (*100*), Indian podophyllum contains 9 to 12 percent of resin (*100*), while podophyllum from *P. sikkimensis* yields 7.5 to 15 percent (*62, 61*) of resin. Podophyllin has a characteristic odor, a bitter, acrid taste, and is very irritating to the eyes and mucous membranes in general.

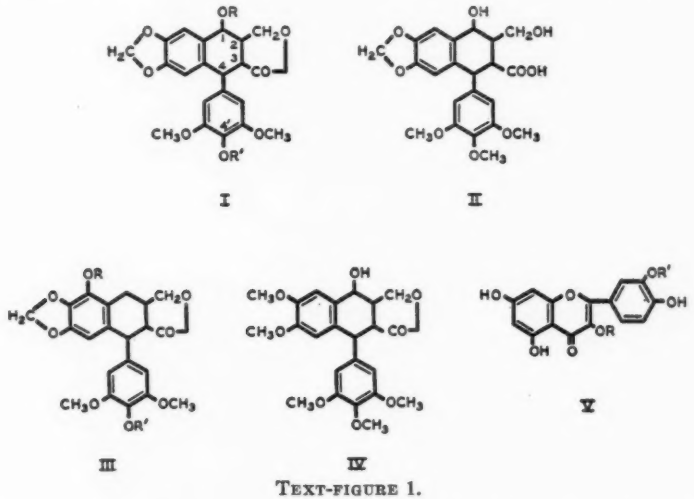
Podophyllin has been reported to contain many substances. Of these, a few have been shown in later work not to be present. Of the remainder, some have been given different names by different investigators. Some are definite crystalline substances, while others are ill-defined mixtures. The following section brings together the pertinent published material and, it is hoped, serves to correct and explain the many errors and contradictions in the literature. The reported constituents are grouped into three classes: *a*) lignans, or substances whose structures are based on 2,3-dibenzylbutane, *b*) pigments, and *c*) other substances.

[9] The Indian plant is here called *P. hexandrum* (Royle), but recently (*58*) this has been classified as a variety of *P. emodi* Wall.

[10] An excellent review of the botany and economics of the *Podophyllum* genus, with special emphasis on the Indian species, has recently appeared (*59*).

A. Lignans

1. *Podophyllotoxin.*—This colorless, crystalline substance was first isolated from American podophyllin and named by Podwyssotzki in 1880 (*273*). Although Kürsten in 1891 (*193*) is always credited with first obtaining the substance in a crystalline state, Podwyssotzki's statement [*273* (a)] that he had definitely observed needle-shaped crystals seems to establish the latter's priority. It was first separated from Indian podophyllin in a noncrystalline form in 1892 by Umney (*351*)[11] and in a crystalline form by Dunstan and Henry in 1898 (*100*).[12] The structure proposed independently by Borsche and Niemann (*36*) and by Späth *et al.* (*319*) was revised by Hartwell and Schrecker (*151*), who proposed formula



TEXT-FIGURE 1.

I (R=H, R′=CH_3), text-figure 1; the four asymmetric carbon atoms imply many stereoisomers of which podophyllotoxin is considered to have the *trans*(1:2)*trans*(2:3)*cis*(3:4) configuration (*151*, *302*).

Podophyllotoxin has a tendency to polymorphism (*354*) and to retain different solvents as solvent of crystallization (*35*, *318*). The different solvates melt between 106° and 118° C. with foaming. The anhydrous form melts at 157 to 158° without foaming (*318*) or 183 to 184° (*151*); these two forms may be dimorphs. Anhydrous podophyllotoxin has $[\alpha]_D$ −109° (*318*) or −111.4° (*344*) in alcohol and −132° in chloroform (*151*). The compound is easily soluble in hot ethanol, ether, chloroform, acetic acid, acetone and hot benzene; sparingly soluble in cold ethanol, cold benzene; and carbon tetrachloride; and almost insoluble in water [0.01% (*200*)] and petroleum ether.

[11] An earlier report by Thompson in 1890 (*343*) unfortunately calls a crude fraction "podophyllotoxin" which is later named "podophyllic acid" (see below).

[12] These authors first showed that podophyllotoxin from American podophyllin was identical with that from Indian podophyllin.

The yield of podophyllotoxin from podophyllin varies with the method of determination used. Tanzen (*341*), investigating several older methods for the estimation of podophyllotoxin involving the isolation of the crude amorphous substance or the conversion of podophyllotoxin present to a crude picropodophyllin, found an average of 40 percent in American podophyllin. Dunstan and Henry (*100*), also using conversion to picropodophyllin, estimated about 20 percent in American and about 38 percent in Indian podophyllin. In modern isolation procedures, these figures for American podophyllin have never been approached. The only ones reported are 9.6 percent of quite pure substance, by Hartwell and Detty (*149*), and 8.5 percent of crude product by Brun (*40*). It is believed that older figures involving isolation of the crude amorphous substance are unreliable because of lack of knowledge of the purity of the product, while those depending on conversion to crude picropodophyllin are high by reason of possible heavy contamination with peltatin derivatives (see below). In the actual isolation of podophyllotoxin from Indian podophyllin, Thompson (*343*) reported 56.6 percent of impure material, Späth *et al.* (*318*) reported 24 to 28 percent of pure crystals, Seshadri and Subramanian (*310*) found 35 percent of pure crystals, and Nadkarni *et al.* (*249*) recorded 47 percent for quite pure material. Since Indian podophyllin apparently contains none of the peltatins (*249*), estimation of podophyllotoxin by conversion to picropodophyllin is not subject to as much error as it is in the case of American podophyllin.[13]

2. *Picropodophyllin.*—This substance was first described by Podwyssotzki [*273* (b)] as having been obtained from podophyllin by the action of slaked lime. Since it has been made clear by later work (*35*, *318*, *293*) that podophyllotoxin is readily converted to picropodophyllin by the action of even weak alkalies, and since picropodophyllin has never been subsequently reported from podophyllin without the agency of alkali, Podwyssotzki's product must be considered an artifact. The compound, in short, is a derivative of podophyllotoxin and is presumed not to exist as such in podophyllin. Despite these considerations, picropodophyllin is still listed erroneously in some of the secondary references (*e.g. 84*, *48*, *234*) as being a component of podophyllin.

Picropodophyllin is a colorless, crystalline compound, melting variously from 215° to 232° C., and having a reported $[\alpha]_D$ in acetone of 9.58° (*318*) and in chloroform of 5.3° (*35*) and 9.38° (*318*). Its accepted structure, as elucidated by Borsche (*35*), Späth (*320*) and their co-workers, is given by the formula for podophyllotoxin (formula I, text-fig. 1; R=H, R′=CH_3). Hartwell and Schrecker believe (*151*, *302*) that the two compounds are stereoisomers, differing only in configuration around C_3; in other words, that picropodophyllin has the *trans*(1:2)*cis*(2:3)*trans*(3:4) configuration.

Due to its close structural similarity to podophyllotoxin, it is of considerable interest for biological research. Because it does not occur

[13] It is of interest to note that savin, the dried needles of *Juniperus sabina* and other junipers, has long been used for the destruction of warts and other excrescences, and for condyloma acuminatum (*323*, *105*, *276*). The crystalline agent in savin and certain other junipers, responsible for the tumor-damaging effect on Sarcoma 37 in mice, has been shown to be podophyllotoxin (*159*).

naturally in podophyllin, however, it will not be considered further here.

3. *Podophyllic acid.*—This term has been used to designate two products: *a*) the hydroxyacid formed by opening the lactone ring of picropodophyllin, and *b*) the fraction of podophyllin obtained by precipitating the chloroform-soluble portion with ether.

The first product is a definite crystalline compound, m. p. 163 to 165° C. with foaming (*35*), having the structure shown in formula II (text-fig. 1). Although it has never been shown to occur naturally in podophyllin, its history is worth recording in order to clarify the literature. Buchheim in 1872 (*46*) treated a noncrystalline, physiologically active fraction of podophyllin with potassium hydroxide and on acidification obtained a yellowish-white, noncrystalline, inactive product which he named podophyllic acid. By treating either podophyllin or podophyllotoxin with ammonia or slaked lime, Podwyssotzki [*273* (a)] similarly obtained, beside picropodophyllin, a colorless, noncrystalline, acidic, nonpurgative substance which he called also podophyllic acid; on attempted crystallization from alcohol, some picropodophyllin was always formed and it is probable that Podwyssotzki's podophyllic acid contained some picropodophyllin. This acid was easily soluble in alcohol, chloroform, and ether; somewhat soluble in hot water; and insoluble in cold water. In another publication, Podwyssotzki [*273* (b)] referrred to this substance as picropodophyllic acid; this is the basis for the listing, sometimes seen in the literature, of picropodophyllic acid as one of the components of podophyllin. From a chloroform extract of podophyllin, Kürsten (*193*) isolated a sparingly soluble barium salt by means of barium hydroxide solution. The free acid could be repeatedly dissolved in ammonia and precipitated with acetic acid, and was obtained as fine soft needles, m. p. 156 to 158°, soluble in alcohol, chloroform and ether. Kürsten called his product picropodophyllic acid. Dunstan and Henry (*100*) prepared the acid, for which they retained the name podophyllic acid, from crystalline podophyllotoxin but were never able to obtain it in crystalline form, all attempts yielding picropodophyllin. They did prepare several metallic salts of the acid and noted the ready crystallizability of the sodium salt. The aqueous solution of the sodium salt was found to be levorotatory. They also prepared the sodium salt of the acid from picropodophyllin and showed that the optical rotation was identical. Tanzen (*341*) was able to prepare podophyllic acid in a crystalline form, m. p. 158°. This product was readily soluble in alcohol, more difficultly soluble in warm water and ether, and difficultly soluble in cold water. Borsche and Niemann (*35*) obtained the crude acid in nearly theoretical yield from either podophyllotoxin or picropodophyllin. On recrystallization from a water-chloroform mixture (*sic*), it was obtained as white needles, melting at 163 to 165° with foaming; at the melting point it was converted into picropodophyllin. Its optical rotation in absolute alcohol was $[\alpha]_D^{16} = -102.8°$ (*c*,1.6). They also prepared the crystalline sodium salt and found the optical rotation in water to be $[\alpha]_D^{18} = -86.7°$ (*c*,0.81) (from podophyllotoxin) and $-85.3°$ (*c*,0.95) (from picropodophyllin).

The second product called podophyllic acid occurs naturally in podophyllin. It has never been obtained in crystalline form, no analyses have been reported on it, and it has been found to be a mixture of substances. Lewis in 1847 (*204*) stated there were two resins in podophyllin, one soluble in alcohol-ether, the other soluble only in alcohol. Bentley in 1861 (*30*) termed the ether-insoluble resin "podophyllic acid." Podwyssotzki [*273* (b)] obtained a brown, resinlike substance which separated out when ether was added to a chloroform extract of American podophyllin. The product, which he called podophyllic acid, was inactive physiologically. It was soluble in alcohol and chloroform, and insoluble in ether, petroleum ether, or water. Thompson (*343*) defatted Indian podophyllin with benzine, extracted the residue with chloroform, poured the extract into ether, and obtained a yield of 57 percent (based on podophyllin before defatting) of a precipitate which he unfortunately called podophyllotoxin. No physical data are given on this preparation. Umney (*351*) similarly isolated podophyllic acid from American and Indian podophyllin, obtaining a yield of 6.9 percent from the American and 30.8 percent from the Indian drug. The latter melted at about 125° C. Mellanoff and Schaeffer (*232*), using a method like that of Thompson, isolated a product from American podophyllin which they called podophyllinic acid. After purifying by dissolving in chloroform and precipitating with ether, the product (yield not given) melted at 159 to 161°, and had an optical rotation in alcohol of $[\alpha]^{25.5} = -104°$ (c,0.5). It was soluble in 95 percent alcohol, chloroform, carbon tetrachloride, and acetone; and sparingly soluble in ethyl acetate, benzene, ether, and water. Finally, using a similar procedure, Viehoever and Mack (*354*) also obtained this product from American podophyllin. The light-brown product so obtained could be decolorized by dissolving in isoamyl alcohol, treating with animal charcoal, and precipitating with petroleum ether. The amorphous substance (no yields stated) melted at 150° (sl. dec.) and had a rotation in alcohol of $[\alpha]^{19} = -80.4°$. It was soluble in alcohol and acetone, slightly soluble in ether, and practically insoluble in petroleum ether and water. The substance was not laxative to *Daphnia*. In unpublished work from this laboratory [*cf.* (*200*)], it has been found that this fraction prepared from American podophyllin essentially according to the procedure of Mellanoff and Schaeffer (*232*), contained a significant portion of the tumor-necrotizing potency of the podophyllin. Chemical evidence for the presence of podophyllotoxin, α-peltatin, and picropodophyllin glucoside was obtained and the first two compounds accounted for all the biological activity of the fraction.

4. *α-Peltatin.*—This compound (formula III, text-fig. 1; R=R′=H) is one of the tumor-necrotizing components of American podophyllin in which it occurs to the extent of 5.5 percent (*147*, *149*, *152*); none is found in Indian podophyllin (*250*, *249*). The only point of doubt in its structure is the position of the lactone group on the hydroromatic ring (*303*). It forms colorless prisms from ethanol, m. p. 230.5 to 232.5° C. cor.; $[\alpha]_D$ −115° (ethanol), −120° (chloroform), −96° (acetone), −160°

(0.1 *N* sodium hydroxide). It is soluble in ethanol, methanol, chloroform, acetone, acetic acid, carbon tetrachloride, propylene glycol, and dilute sodium hydroxide solution; less soluble in benzene and ether; and practically insoluble in petroleum ether and water. Although, like podophyllotoxin, α-peltatin is epimerized by even weak alkalies, a solution in 0.05 *N* sodium hydroxide will retain a large part of its biological activity for 24 hours.

5. *β-Peltatin.*—This compound is (with podophyllotoxin and α-peltatin) one of the three tumor-necrotizing agents found in American podophyllin. It occurs to the extent of 5.7 percent (*148*, *152*); none is found in Indian podophyllin. Chemically, it is the 4′-monomethyl ether of α-peltatin; it is expressed by formula III in text-figure 1 (R=H, R′=CH_3). It forms colorless prisms, m. p. 231.1 to 238.0° C. cor.; $[\alpha]_D$ −115° (ethanol), −119° (chloroform), −95° (acetone), and −141° (0.1 *N* sodium hydroxide). The solubilities and stability of β-peltatin are similar to those given above for α-peltatin.

6. *4′-Demethylpodophyllotoxin.*—This compound is (with podophyllotoxin) one of the two found in Indian podophyllin that produced damage in animal tumors (*250*, *249*). It occurs to the extent of 1.7 percent and has not been found in any other variety of podophyllin. It forms colorless, transparent prisms from ethanol, m. p. 250.0 to 251.6° C. cor.; $[\alpha]_D$ −130° C. (*c*, 0.75, chloroform). Its structure (formula I, text-fig. 1; R=R′=H) was deduced from the fact that methylation with diazomethane yielded podophyllotoxin, while oxidation of the phenolic ethyl ether gave syringic acid ethyl ether. 4′-Demethylpodophyllotoxin is fairly soluble in chloroform, acetone, hot ethanol, and dilute caustic alkali; it is practically insoluble in water, hexane, cold methanol, or cold ethanol.

7. *Sikkimotoxin.*—The only reported study of the resin from *P. sikkimensis*, by Chatterjee and his associates (*62*, *61*), has revealed a new lignan in this species, sikkimotoxin. Other lignans appear to be absent. Sikkimotoxin has been isolated (*61*) in 47 percent yield as colorless crystals, m. p. 120° C. (effervescence); $[\alpha]_D$ −91.9° (ethanol), −92.1° (chloroform) (*60*). It is highly soluble in ethanol, methanol, and chloroform; moderately soluble in isobutyl alcohol and boiling benzene; sparingly soluble in cold benzene; insoluble in water, petroleum ether, and ether. Chemical studies (*61*, *60*) make it appear probable that sikkimotoxin is related to podophyllotoxin configurationally and has the structure represented by formula IV (text-fig. 1).[14]

8. *Picropodophyllin glucoside.*—This substance, which is the first lignan glycoside found in a plant of the *Podophyllum* genus, occurs in Indian podophyllin to the extent of 1.8 percent (*250*, *249*), and there is evidence that it occurs in American podophyllin (see above under *Podophyllic Acid*). It is inactive against Sarcoma 37 in mice. It forms long, thin,

[14] In unpublished experiments of Leiter and Hartwell, a sample of the resin of *P. sikkimensis* kindly provided by Dr. Chatterjee showed as high activity in damaging Sarcoma 37 in mice as did the resins from the other species of *Podophyllum*. This makes it seem probable that sikkimotoxin is the component of the resin responsible for the activity.

colorless needles from 75 percent methanol or from water, analyzing for a hemihydrate, m. p. 237.0 to 238.2° C. cor.; $[\alpha]_D - 11.5°$ (c,0.5, pyridine). It is slightly soluble in boiling methanol, boiling water, and pyridine; and practically insoluble in cold methanol, water, acetone, chloroform, ethyl acetate, and cyclohexane. Chemical studies revealed its structure to be that represented by the systematic name, 1-0-(β-D-glucopyranosyl)-picropodophyllin (formula I, text-fig. 1; R=glucosyl, R′=CH_3).

B. Pigments

1. *Quercetin.*—At least part of the color of podophyllin is due to the presence of a pigment readily crystallizing in yellow needles, which has been usually called podophylloquercetin. The name was apparently given to it by Podwyssotzki [*273* (a)], who recognized its close similarity to quercetin in analysis, melting point, and certain physical and chemical properties. Although Kürsten (*193*) thought that podophylloquercetin was not identical with quercetin from quercitron bark, Dunstan and Henry (*100*) completely purified the pigment from both varieties of podophyllin and seem to have settled the identity with quercetin. The latter workers prepared the crystalline acetate and methyl ether and found melting points identical with the corresponding derivatives of quercetin; the crystalline sulfuric acid addition products had the same composition and both pigments yielded the same degradation products on alkali fusion. Its structure is represented by formula V in text-figure 1 (R=R′=H).

Quercetin is soluble in hot alcohol, hot acetic acid, ethyl acetate, and aqueous solutions of alkalies; sparingly soluble in cold alcohol, cold acetic acid, and ether; and very difficultly soluble in chloroform and water. Because of its chloroform insolubility, quercetin is found in the podophyllin residue after removing the other components with chloroform. It has been extracted from this residue with ether by Podwyssotzki [*273* (a)], Kürsten (*193*), Umney (*351*), Dunstan and Henry (*100*), and Mellanoff and Schaeffer (*232*); of these, only Umney gives yields, namely, 2.4 percent from American podophyllin and 1.3 percent from Indian podophyllin. Seshadri and Subramanian (*310*), using a simplified method of isolation, found 7 percent of the pure substance in Indian podophyllin. Recently (*62*), quercetin has been obtained from the resin of *P. sikkimensis* in yields not stated.

2. *Methylpodophylloquercetin.*—Wehmer (*366*) erroneously cites Kürsten (*193*) as having reported this compound in podophyllin. Actually, Kürsten prepared a methylpodophylloquercetin as a derivative from the quercetin he isolated from podophyllin, and never claimed it as a constituent of podophyllin.

3. *Isorhamnetin.*—This flavone pigment has been identified in the resin of only one species of *Podophyllum*, namely *sikkimensis* (*62*). It was found in the ether-soluble part of the chloroform-insoluble fraction of the resin, to the extent of less than 0.02 percent. It has the structure represented by formula V in text-figure 1 (R=H, R′=CH_3).

4. *3-Galactosidylquercetin.*—This flavone glycoside was also found in the resin of *P. sikkimensis*, accompanying quercetin and isorhamnetin (*62*). It was isolated from the ether-insoluble part of the chloroform-insoluble fraction. Galactose and quercetin were identified in the hydrolysate, and the m. p. of 237° C. agreed with that reported (*301*) for a sample isolated from the skins of apples. Its structure is given by formula V in text-figure 1 (R=galactosyl, R′=H).

5. *Substance melting at 140 to 143° C.*—From the mother liquors of the methanol recrystallization of podophyllotoxin obtained from the chloroform-soluble portion of Indian podophyllin, Borsche and Niemann (*35*) isolated a small amount of stout, yellow prisms, m. p. 140 to 143° C. Since the substance was not further studied, and no analysis was given, it is not known whether the product was a single chemical individual or if it was new.

6. *Berberine.*—Since *Podophyllum* is one of the family of Berberidaceae, the presence of berberine was early sought for and was reported in podophyllum, along with a colorless alkaloid, by Mayer (*230*). The colorless alkaloid was also said to be in the resin. Berberine was first believed to be responsible for the color of podophyllin. The work of Power (*277*, *278*), Maisch (*224*), Podwyssotzki [*273* (a)], and others proves that berberine and other alkaloids are absent in American podophyllum and its resin, and that Mayer's positive test was illusory. Occasional positive tests reported for alkaloids must have been due to contamination of the plant material, probably with *Hydrastis* (*225*). This inference is supported by the method of preparation of podophyllin. In this country, the practice is to precipitate the podophyllin by drowning the alcoholic extract of the root in water acidified with hydrochloric acid, a process which would remove basic substances.

C. Other Substances

1. *Podophylloresin.*—Dunstan and Henry (*100*) found that the residue from podophyllin, after exhaustive extraction with chloroform and with ether, was still very active as a purgative. The only solvent found for this residue was alcohol. By fractionally precipitating the alcohol solution with water, the residue was separated into two fractions. One was a soft, transparent, brownish-red resin; the other was a black powder almost insoluble in alcohol. The first was active as a purgative and was called podophylloresin; the second was quite inactive. When the podophylloresin was treated with acetic anhydride, a colorless, crystalline substance was obtained, melting constantly at 198° C. This proved to be a diacetate and, according to the analysis, the resin was given the formula $C_{12}H_{12}O_4$ or $C_{12}H_{10}O_2(OH)_2$. Potash fusion of the resin yielded protocatechuic acid and probably *p*-hydroxybenzoic acid.

Recently, Seshadri and Subramanian (*310*) have undertaken a study of podophylloresin and have obtained it by an improved method in a yield of 8 percent from Indian podophyllin. When an acetone solution of podophyllin, filtered to remove a small amount of inorganic matter, was treated

with chloroform, a sticky resin slowly separated. After the resin was taken up in acetone and precipitated with ether, it was dissolved in sodium bicarbonate or hydroxide solution, precipitated with acid, and the precipitate finally precipitated from alcohol solution with ether. It was obtained as a pale brown microcrystalline powder, m. p. about 115° C. (dec.), and renamed "podophyllol" to avoid confusion with resin of podophyllum. Analysis was consistent with the formula $C_{20}H_{22}O_8 \cdot H_2O$. Positive tests were obtained for the phenolic hydroxyl group and the methylenedioxy group. Acetylation (acetic anhydride and sodium acetate) and methylation (dimethyl sulfate) both yielded mono-derivatives the acetate being readily crystalline and the methyl ether microcrystalline. The monoacetate contained one methoxyl group and was levorotatory. In view of these properties, podophyllol was believed to consist essentially of a lignan related to podophyllotoxin.

2. *High molecular weight fraction.*—A high molecular weight fraction, possibly a complex polysaccharide, has been reported (*211–213*) from podophyllin (source not stated). Isolation was by a process involving dialysis, centrifugation, and electrophoresis. The fraction was stated to have an oncolytic effect on certain tumors in mice.

3. *Oil, fatty substances.*—Power (*278*) obtained a fatty substance in quite well-defined crystals when a concentrated alcoholic solution of podophyllin was allowed to stand for some time at a low temperature. No melting point or other property was determined but the probability was expressed that it might be myristic acid. Podwyssotzki [*273* (a)] extracted podophyllin exhaustively with chloroform, removed the solvent, and extracted the chloroform-soluble residue with petroleum ether. By evaporation of the petroleum ether, he obtained a dark green paste which after some time deposited crusts of a colorless fatty acid which crystallized in rectangular plates resembling cholesterol. The mother liquor consisted of a green oil with a disagreeable odor. Neither the fatty acid nor the oil had purgative action and neither was further investigated. No yields were given. Thompson (*343*) obtained a 4-percent yield of benzine-soluble material from Indian podophyllin. Umney (*351*) reported the presence of 5.7 percent fatty matter in American podophyllin and 2.3 percent in the Indian variety; that from the former was distinctly crystalline, while that from the latter was noncrystalline and semifluid. Dohme and Engelhardt (*95*) investigated a green oil obtained by extracting American podophyllum with a "suitable solvent"; fatty acids were obtained from the saponifiable fraction and two crystalline sterols or sterol-like substances from the unsaponifiable fraction. This work may not be pertinent to the present review, however, as it is not clear what relationship this oil bears to that obtained from podophyllin. According to Tanzen (*341*), a phytosterol-like compound is present in podophyllin. Finally, Mellanoff and Schaeffer (*232*) extracted American podophyllin with petroleum ether. Removal of the solvent yielded a fatty substance, yield not stated, that melted at 24.5° C. It showed birotation and contained 24.42 percent of unsaponifiable matter.

4. *Saponin.*—Saponin was early mentioned as a constituent of podophyllin. Its presence has never been confirmed.

5. *Glucoside.*—Guaresci (*142*, *143*) first reported the presence of a glucoside in podophyllin. It could not be isolated but its presence was inferred from the fact that the ether-insoluble fraction of podophyllin yielded glucose on hydrolysis with either dilute sulfuric acid or with emulsin. The aglucon was not studied. No other glycosides were reported in podophyllin until the actual isolation of two of them, 3-galactosidylquercetin (*q. v.*) and picropodophyllin glucoside (*q. v.*).

6. *Residue.*—After extraction of podophyllin with chloroform and ether, and removal of podophylloresin, a residue is obtained (*100*) which is quite inactive as a purgative. It consists of a black powder, almost insoluble in alcohol and containing calcium and magnesium.

Summary

A summary of the chemistry of podophyllin shows that resins derived from different species of *Podophyllum* differ in composition. For instance, podophyllotoxin occurs in *P. peltatum* and *P. emodi* but has not been reported in *P. sikkimensis*; the peltatins are apparently characteristic of *P. peltatum*; demethylpodophyllotoxin has been found only in *P. emodi*; while sikkimotoxin has been reported only in *P. sikkimensis*. As far as has been studied, there are differences in other components, also, such as pigments. Table 1 lists the well-characterized components of podophyllin along with the species of podophyllum in which they have been reported.

TABLE 1.—*Well-characterized components of resin from Podophyllum species*

Name	Present in		
	P. peltatum	*P. emodi*	*P. sikkimensis*
Podophyllotoxin	+	+	
α-Peltatin	+		
β-Peltatin	+		
4′-Demethylpodophyllotoxin		+	
Sikkimotoxin			+
Picropodophyllin Glucoside	+	+	
Quercetin	+	+	+
Isorhamnetin			+
3-Galactosidylquercetin			+

Podophyllin from any source is a complex mixture chemically which may, on further study, yield other substances of whose existence we are not yet aware. The implications of these facts for biological and clinical research seem to be clear, namely, that in working with this material due account should be taken of its nonhomogeneity and nonuniformity of composition. The pure compounds derived from the different podophyllins should be of increasing interest in research of many kinds.

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